Astrocytes: Targets for neuroactive substances

Thesis

ASTROCYTES - TARGETS FOR NEUROACTIVE SUBSTANCES

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THESIS SUBMITTED IN PART FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY FROM THE OPEN UNIVERSITY, MAY 1985

Author's number : HDK 64229

Date of submission : 23 May 1985

Date of award : 10 October 1985

Fibronectin positive cells, probably derived from the meninges, were found to be the major contaminant of astrocyte-enriched cultures.

4) Astrocytes and meningeal cells in vitro contained glycogen and both cell cultures responded to changes in the extracellular K^+ concentration ($[K^+]_o$) by alterations in glycogen metabolism. The effects of changes in $[K^+]_o$ on astrocyte and meningeal cell culture glycogen content were different. Astrocytes responded to smaller changes in $[K^+]_o$ than did meningeal cell cultures.

5) Membranes prepared from astrocyte-enriched cultures possessed binding sites for three neurotransmitter receptor ligands [^{3}H]-dihydroalprenolol, [^{3}H]-serotonin and [^{3}H]-quinuclidinyl benzilate. Membranes prepared from meningeal cell cultures also possessed binding sites for [^{3}H]-dihydroalprenolol and [^{3}H]-serotonin but not for [^{3}H]-quinuclidinyl benzilate.

6) Noradrenaline, but not serotonin or carbachol reduced the glycogen content of astrocyte-enriched cultures. Compounds which raised the level of intracellular cAMP without interaction with neurotransmitter receptors, e.g. forskolin, also induced glycogenolysis in astrocyte cultures. Noradrenaline and forskolin also promoted net glycogen breakdown in meningeal cell cultures.

7) Selective α- and β-adrenoreceptor agonists phenylephrine and isoproterenol respectively did not evoke changes in the net glycogen content of astrocyte-enriched cultures. In contrast, both of these compounds caused net glycogenolysis in meningeal cell cultures.

8) The action of noradrenaline on cell culture glycogen stores was not blocked by the α- and β-adrenoreceptor antagonists phentolamine and propranolol respectively, though these compounds themselves caused glycogenolysis. More selective α-adrenoreceptor subtype antagonists did not block the slight effect of phenylephrine on

astrocyte glycogen content. In the case of yohimbine, an α_2-adrenoreceptor antagonist, the glycogenolytic action of phenylephrine was potentiated. Prazosin, an $\alpha_1$adrenoreceptor antagonist, markedly reversed the action of phenylephrine on meningeal cell glycogen content.

9) Reserpine treatment of astrocyte-enriched cultures caused a marked increase in glycogen content and in the efficacy of serotonin to evoke glycogenolysis. In contrast, reserpine treatment of meningeal cell cultures caused a net loss of glycogen.

ACKNOWLEDGEMENTS

There are many people who have helped directly or indirectly in the completion of this thesis and to whom I would like to express my gratitude for their assistance and friendship. Firstly, I would like to thank my supervisor Sean Murphy, for taking me on in the first place and for his continued support and encouragement over the course of my studies. Great thanks are also due to Brian Pearce for his help in the lab. and in the formulation of this thesis. Excellent technical and moral support was provided by Chrissie Morrow and Jill Grimble.

Other who have helped me in a variety of ways both within and outside the lab. include Kieth Elcoate (for sharing the flat, insults and hangovers), Barney and particularly Adrian (for losing the bet), Mo and Bo (in their own ways), Caroline and Val, John Rudge Paul Gabbott, my family (for their support whilst I was "between degrees" and not pressing me too hard to get a "proper" job), Steve Walters and all in the animal house, Judith Eastburn (for much appreciated help with the photography), John Tilzey, Paul Verrell, Norah McCabe, Paul Rose, Julie Roberts, Marion and Philip, Sue Harding Penny Tillson, Chris Mattacks, Jane Wild, Bill and Margret Markland, Dougie Wilson and also the O.U.C.C. and Bletchley R.U.F.C. (for taking my mind off astrocytes).

I am grateful to Prof. M. Raff and Drs. G. Wilkin, B. Anderton, A. Williams, R. Morris, B. Ranscht and G. Pilkington for gifts of antisera. I would also like to acknowledge grants from the O.U. and E.S.F. for my project, conferences and an E.T.P. training course.

Last, but by no means least, I would like to thank Elaine Dale for her rapid and accurate typing of the manuscript.

ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AG	Amylo-α 1,4-α 1,6-glucosidase
BSA	Bovine serum albumen
CNPase	Cyclic nucleotide phosphohydrolase
CNS	Central nervous system
dBcAMP	Dibutyryl cyclic adenosine monophosphate
2-DG	2-Deoxyglucose
2-DG-6-P	2-Deoxyglucose-6-phosphate
DHA	Dihydroalprenolol
d.i.v.	Days in vitro
d.p.m.	Disintegrations per minute
FCS	Foetal calf serum
FN	Fibronectin
GC	Galactocerebroside
GFAP	Glial Fibrillary acidic protein
GGT	γ-Glutamyl transpeptidase
GPa	Glycogen phosphorylase (active)
GPb	Glycogen phosphorylase (inactive)
GPDH	Glycerol phosphate dehydrogenase
GSa	Glycogen synthetase (active)
GSb	Glycogen synthetase (inactive)

5-HT	Serotonin
MSH	Melanocyte stimulating hormone
NGS	Normal goat serum
PBS	Phosphate buffered saline
PG	Prostaglandin
QNB	Quinuclidinyl benzilate
r.p.m.	Revolutions per minute
s.e.m.	Standard error of the mean
SOM	Somatostatin
SP	Substance P
SV	Subventricular zone
TBS	Tris buffered saline
UDP-glucose	Uridine diphospho-glucose
VIP	Vasoactive intestinal polypeptide

LIST OF PUBLICATIONS

Some of the data presented in this thesis has been presented elsewhere:-

CAMBRAY-DEAKIN, M.A., MORROW, C. AND MURPHY, S. (1985), Cortical astrocytes in primary culture: presence of neurotransmitter binding sites. Biochem. Soc. Trans. 13, 231-232

PEARCE, B., CAMBRAY-DEAKIN, M. AND MURPHY, S. (1985a), Glial glycogen stores are regulated by α-adrenergic receptors. Biochem. Soc. Trans. 13, 232-233

PEARCE, B., CAMBRAY-DEAKIN, M. AND MURPHY, S. (1985b), Astrocyte opioid receptors: activation modifies the noradrenaline-evoked increase in 2-[^{14}C] deoxyglucose incorporation into glycogen. Neurosci Lett. 55, 157-160

PEARCE, B., CAMBRAY-DEAKIN, M. AND MURPHY, S. (1985c), Activation of muscarinic and α_1-adrenergic receptors on astrocytes results in the accumulation of inositol phosphates. J. Neurochem. 45,

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CHAPTER 1

General Introduction

The 19th century anatomist Virchow was the first to divide the cells of the mammalian central nervous system (CNS) into two classes, the neurons and neuroglia (see Penfield, 1932; Privat, 1975; Sturrock, 1975; Privat and Fulcrand, 1977 for historical reviews). The early microscopists, some using the techniques of metallic impregnation of nervous tissue pioneered by Golgi (e.g. Golgi, 1885), further divided the neuroglia into subclasses, the macroglia comprised of astrocytes and oligodendrocyes, and the microglia (Weigert, 1895; Ramon y Cajal, 1909; Rio-Hortega, 1919). This thesis is primarily concerned with the biochemistry of one class of neuroglial cell, namely the cerebral cortical astrocyte. These cells are numerous, constituting approximately 15 - 20% of the total number of cells present in the cerebral cortex (Parnavelas et al, 1983). Until recently, they have been generally considered to constitute a packing tissue in the brain or to contribute to the blood-brain barrier (e.g. Peters et al, 1976). Recent evidence, such as the presence of neurotransmitter receptors linked to second messengers on astrocytes (see review by Van Calker and Hamprecht, 1980), has suggested a more dynamic role for astrocytes in CNS function.

Since the early observations, extensive investigations using the light and electronmicroscope have resulted in a detailed analysis of the morphology and distribution of neuroglia in the adult and developing brain, a synopsis of which is given here (see Peters et al, 1976; Palay and Chan-Palay, 1977; Privat and Fulcrand, 1977; Parnavelas et al, 1983).

Astrocytes in the adult mammalian brain have a rounded perikaryon of about 9 - 10 μm in diameter. The cytoplasm characteristically contains glycogen granules and 9 - 10 nm diameter filaments which penetrate into the large number of branching processes which radiate from the cell body. Some of the processes form terminal expansions (end feet) which are applied to the surfaces of blood vessels, or are associated with a basement membrane and constitute a layer covering the surface of the brain, the glia limitans. Astrocytes are abundant in all parts of the CNS although their fine structure and morphology varies between regions. In the grey matter, astrocytes, whilst retaining the basic morphology previously described, generally contain fewer glial filaments than those in the white matter. Thus, following the original work by Weigert in 1895, grey matter astrocytes are termed protoplasmic and those in the white matter, fibrous. However, it must be noted that the difference in filament content between the two cell types is not always evident. For example, the "protoplasmic" astrocytes of the cerebellar grey matter (Golgi epithelial cells or Bergmann glia) contain few filaments whilst the "protoplasmic" astrocytes of the inferior olive are very similar to fibrous astrocytes in this respect (cf. Peters et al, 1976).

Oligodendrocytes are often described as more phase dark or electron dense cells than astrocytes when viewed by the light microscope or electronmicroscope. They do not contain glial filaments or glycogen granules and have finer, more delicate processes than astrocytes. The processes may contain myelin and wrap round some neuronal processes to form a myelin sheath.

Microglial cells are smaller than astrocytes or oligodendrocytes,

often appearing more dark under the microscope than oligodendrocytes as the nucleus, containing heavy clumps of chromatin, constitutes most of the cell volume. Again, like oligodendrocytes, they contain no glycogen or glial filaments in the cell body or in their rather broad processes.

Macroglia and microglia, whilst both being termed neuroglial cells, have separate embryological origins. Microglial cells are mesodermal cells and are thought to be derived from circulating monocytes which penetrate the nervous system from the vascular system early in development (see Ling, 1981, for review). In contrast, macroglial cells are neuroepithelial cells derived from the subventricular zone (SV) of the developing neural tube, although the exact course of their development is unclear.

Initially, His (1889) suggested that neurons and neuroglia were formed from separate precursor cells dividing in the SV. However, Schaper (1897) proposed an alternative view, that the precursor cells in the SV were indeterminate and gave rise to cells which migrated out to the intermediate zone of the developing brain where they divided to yield neurons and neuroglia. Subsequently, Sidman et al (1951) and Fujita (1963; 1966) proposed that the mitotically active precursor cells in the SV first gave rise to neurons and then to neuroglial cells. However, glial cells, in particular the radial glial cells and Golgi epithelial cells whose processes span the width of the developing brain, were seen to be present at early stages during neurogenesis (Ramon y Cajal, 1911; Rakic, 1971; Antanitus et al, 1976; Levitt et al, 1983). It also became clear that a distinct population of dividing glial cell precursors co-existed with neuronal precursors (Skoff, 1980;

Rakic, 1981; Levitt et al, 1981; 1983). Therefore, it was proposed that at this stage neurons and glia were derived from separate stem cells as originally stated by His. Radial glia are thought to divide later in development (Schmechel and Rakic, 1979) and differentiate into mature astrocytes constituting some, but not all, of those found in the brain (see Rakic, 1981, for review also Pixley and de Vellis, 1984). Some oligodendrocytes are also believed to be formed from radial glia, as suggested by the electron-microscopic and immunohistochemical studies of Choi et al (1983). Oligodendrocytes and one type of astrocyte, derived from the optic nerve, do indeed share a common precursor cell (Raff et al, 1983, 1984; Temple and Raff, 1985), although it is not known whether this common precursor cell represents a radial glial cell. The differentiation of the macroglia is, as previously stated, not fully understood and is presently the subject of intensive examination in several laboratories.

Since the early division of CNS cells into neurons and neuroglia it has become axiomatic that neurons are involved in the signal processing function of the CNS, whilst to the neuroglia have been ascribed a variety of supportive roles. Thus oligodendrocytes are considered to be responsible for the laying down of insulating myelin sheaths around some CNS neurons, analogous to the function of Schwann cells in the peripheral nervous system (see Johnston and Roots, 1976; Palay and Chan-Palay, 1977). Microglial cells have been assigned a role as CNS macrophages, removing damaged or effete material from the brain (Vaughn and Peters, 1974; Ling, 1981).

Due to the highly complex nature of the mammalian cerebral cortex, many of those examining the function(s) of differentiated CNS glial

cells have sought to obtain pure or enriched populations of astrocytes for study. Three methods have been used; the bulk separation of cells from the adult cortex, the primary culture of cells (usually derived from the neocortex), or the use of clonal cell lines. The two former methods are reviewed more fully later (Chapter 2 and 3). Bulk isolation of astrocytes might be considered to be an ideal model for astrocytes in situ. Theoretically such a procedure should yield metabolically active, identifiable and intact cells with little contaminating material from other cell types. It would have an advantage over in vitro preparations in that the astrocyte population would be obtained from a mature brain and thus be fully differentiated. This is unlike the growth of astrocytes in culture where the cells are derived from undifferentiated (perinatal) brain, maintained as a monolayer and, in the most part, in the absence of other cell types. The development of astrocyte function in vitro might therefore not follow the same course as that in vivo because possible inductive effects from other cell types may be removed. Similarly, clonal cell lines, i.e. cells derived from a naturally occuring or artificially produced tumour (transformed cells), may not express the same characteristics as non-transformed astrocytes in vivo (see Pfeiffer, 1978, for review). In addition, cell lines are distinct from astrocytes in vivo and those in primary culture in that they possess the ability to divide continually over many cell cycles and, as such, constitute an immortal cell line. Furthermore, the most commonly used glial cell line, C6, induced in a rat by N-nitrosomethylurea in 1968 (Benda et al, 1968), possesses mixed glial properties. For example, it not only expresses the astrocyte specific protein S-100 (Benda et al, 1968) but also the oligodendrocyte specific enzyme glycerol phosphate dehydrogenase (see Van Calker and Hamprecht, 1980).

In spite of the interpretative problems associated with the use of primary cultures of astrocytes and glial cell lines, they have been used extensively as models for differentiated astrocytes in vivo, in the large part due to the numerous technical difficulties inherent in bulk isolation methods. It must however be borne in mind that they may not entirely reflect the astrocyte in vivo and, where possible, comparisons must be made to the behaviour of astrocytes in situ.

A wide variety of functions have, however, been suggested for the astrocyte based on experiments using the techniques described above. Some of these roles are seen as either bio-chemically or structurally supportive to the functions of neurons. For example, the clearance of potassium (K^+) released from active neurons into the extracellular space (Walz and Hertz, 1984; see also Chapter 4 of this thesis), or the guidance of migrating neurons (Rakic, 1971 et seq.). However, the wide range of proposed functions suggested for astrocytes do not fall readily into categories. For example the well documented ability of astrocytes to inactivate a wide range of neurotransmitters (Henn and Hamberger, 1971; Schousboe et al, 1976; Hertz, 1979; Kimelberg and Pelton, 1983; Hansson, 1983; Inoue et al, 1984) overlaps, in the case of glutamate, with the removal of toxic ammonia. Thus the astrocyte specific enzyme glutamine synthetase combines glutamate and ammonia to form glutamine (Sadasivudu et al, 1977; Norenberg and Martinez-Hernandez, 1979). Similarly, the postulated release of trophic factors from astrocytes which influence the growth and division of neurons (Rudge et al, 1985) may overlap with a more direct metabolic support of neurons through the transfer of metabolic intermediates. For example, Selak et al (1984) suggest

that a transfer of pyruvate from astrocytes to neurons may constitute part of the trophic action of astrocytes on neurons. Shank and Campbell (1981; 1982; 1984a,b) and Yu et al (1983) have proposed that astrocytes may release pyruvate, α-ketoglutarate and malate, to be taken up and utilised by neurons as an energy source or to replenish amino-acid neurotransmitter pools.

Thus astrocytes are generally viewed as nurse cells for neurons (Johnston and Roots, 1976). Indeed, astrocytes are localised between the neuron and its ultimate source of energy which is glucose derived from the blood stream (Sokoloff, 1977; Siesjo, 1978). One characteristic feature of astrocytes is the abundance of the glucose polymer glycogen. Moreover, the astrocyte is intimately associated with the surface membrane of the neuron in situations where the cell or its axon are free from myelin (Wolff, 1970; Johnston and Roots, 1976; Peters et al, 1976; Palay and Chan-Palay, 1977). Indeed Sotelo and Palay (1968) showed that in Deiter's nucleus, astrocyte processes were closely applied between the neuron and the myelin sheath. Thus astrocytes situated between, and in close conjunction with, both blood supply and neuron are ideally situated to fulfil a nutritive or nurse cell role for neurons (Wolff, 1970).

In invertebrates a role for neuroglial cells in the maintenance of energy supplies to neurons has also been proposed recently by Pentreath and co-workers (Pentreath, 1982; see also Chapter 4 of this thesis). Glycogen stored in glial cells in the leech Haemopsis sanguisuga and the snail Planorbis corneus is proposed to be a major store of energy which is utilised by the neuron during periods of increased activity. Furthermore, it is suggested that the turnover

of glial glycogen is under the control of neurons via changes in extracellular K^+ ($[K^+]_o$) which are taken to constitute signals between neurons and glia.

Previously it had only been suggested or presumed that the various supportive functions of glia were controlled by signals from neurons to glia (Galambos, 1961; Varon and Somjen, 1979). The term signal is used here to describe any factor which is influenced by the behaviour of one cell type (e.g. neurons) and which in turn affects the behaviour of other cell types (e.g. astrocytes). Indeed many reports have shown that astrocytes are responsive to factors (signals) such as K^+, Ca^{2+} or neurotransmitters which might be derived from, or influenced by, neurons (Orkand, 1978; Van Calker and Hamprecht 1980; Latzkavits et al, 1982; Sugino et al, 1984).

Astrocyte processes ramify extensively throughout the CNS and are believed to possess surface areas constituting 10 - 40 cm^2/mm^3 of neuropil, the fine, densely matted cellular processes which fill the "intercellular spaces" of the CNS. This represents a very large receptive field available for any signals which might be released by neurons. In addition the synapse is enclosed and invested by astrocyte processes (Johnston and Roots, 1976; Peters et al, 1977; Palay and Chan-Palay, 1977). There are also reports of "synaptoid" contacts between neurons and glia in the mammalian CNS similar to classical synapses (Guldner and Wolff, 1973; Henrikson and Vaughn, 1974; Peters et al, 1976; Wolff et al, 1979; Edmunds and Parnavelas, 1983) although the reported frequency of such contacts is low. Thus astrocytes in vivo may be exposed to neuronally released neurotransmitters. Moreover, the vertebrate CNS contains several groups of monoamine releasing neurons whose processes extend throughout the cerebral cortex and cerebellum but do not make typical synaptic contacts on other neurons. Such cells may release neurotransmitters over the surface of neurons and glia alike (Lapierre et al, 1973; Moore and Bloom, 1979; Lauder, 1983; Florey, 1984).

In summary, previous biochemical and morphological work has suggested that astrocytes form a link between the neuron and blood supply. This may constitute a system transporting and storing metabolic substrates which may be controlled by signals derived from neurons. In particular, glycogen has been proposed to be a store of metabolic intermediates sensitive to neuronal activity. Indeed the possession of large stores of glycogen is one of the few known biochemical characteristics of astrocytes in vivo. This thesis will examine the effects of various conditions which might be considered to mimic naturally occuring signals released from neurons, on the glycogen stores of rat cortical astrocytes

The remainder of this thesis is divided into discrete chapters, each with introduction, methods, results and discussion sections, except for the last, where general conclusions and the possible future directions of research are discussed. Chapters 2 and 3 describe attempts to produce a model system for astrocytes, first using a previously published technique to separate adult astrocytes from the rat cerebral cortex in bulk. Various modifications of the basic technique are described which were designed to increase the yield and purity of the astrocyte-enriched fraction thus produced. The results from experiments to develop an alternative method for the separation of adult cortical astrocytes by immunaffinity separation are also shown. Due to the unsuitability of the resulting fractions, astrocytes were derived from cultures of neonatal rat brain. The method used for this is described in Chapter 3, together with the extensive characterisation of the cultures by various morphological, immunocytochemical and biochemical means. A method for the culture of meningeal cells is also described as these cells were considered

to be the princip . , if not sole, contaminants of the astrocyte cultures.

Chapter 4 reveals that astrocytes in primary culture do indeed metabolise stores of glycogen. When it had been established that meningeal cells in vitro also contained glycogen, the effects of increased $[K^+]_o$ on astrocyte glycogen stores were examined using the corresponding effects of increasing $[K^+]_o$ on the glycogen content of meningeal cell cultures as control. This work was carried out with the broad aim of determining whether mammalian astrocyte glycogen stores might be affected by increases in $[K^+]_o$, as had been shown in invertebrate glia. This is seen to be so for both cell types, although the net effects of $[K^+]_o$ on meningeal cell glycogen stores are different from those on astrocyte glycogen stores.

Other possible signals between neurons and astrocytes are considered in Chapters 5 and 6. Chapter 5 describes binding studies of three neurotransmitter receptor ligands [^{3}H]-serotonin (serontonin receptors), [^{3}H]-dihydroalprenolol (β-adrenergic receptors) and [^{3}H]-quinuclidinyl benzilate (muscarinic cholinergic receptors) to membranes prepared from cultured astrocytes and meningeal cells. Binding sites for [^{3}H]-serotonin and [^{3}H]-dihydroalprenolol are found on both astrocyte and meningeal cell membranes. However, [^{3}H]-quinuclidinyl benzilate bound to astrocyte but not meningeal membranes. The effects of serotonergic, adrenergic and muscarinic cholinergic receptor agonists and antagonists on stores of glycogen in both cell types are then described in Chapter 6. Transmitter agonists and antagonists both produce effects on astrocyte and meningeal glycogen stores, although the two cell types show different net responses.

Finally, these results and others from the same laboratory are discussed under the broader hypothesis that neuron-glia signalling employs a variety of means, and speculations are made with respect to the extent of the metabolic interactions between neurons and astrocytes.

CHAPTER 2

Bulk separation of astrocytes from adult rats

Introduction

In vivo, the astrocyte is intimately associated with neurons and other cell types. Thus experiments to examine the direct effects of putative signals released from neurons on astrocyte biochemistry require the preparation of an isolated, viable and pure preparation of cells. As previously discussed in Chapter 1, three types of preparation have been commonly used: bulk isolated cells from adult tissue, primary cultures of perinatal cells and clonal cell lines. This chapter describes attempts to separate astrocytes from the adult rat cerebral cortex.

A variety of techniques for the isolation of astrocytes from the adult mammalian brain have previously been used in biochemical studies of these cells. The first attempts at the isolation of CNS cells employed free-hand microdissection of Deiter's nucleus to separate neurons and glial cells, although the yield of cells limited any extensive biochemical examination (e.g. Hyden, 1959). Korey (1957) reported that glial cells could be purified in bulk from bovine white matter using differential density centrifugation and sucrose gradients. This preparation contained cellular debris and other cell types (Fewster et al, 1976) and more recent work began with the reports of Rose (1965, 1967) which described the separation of neurons and glia (neuropil) from the rat cortex. This system, primarily designed to yield

neuronal cells, used a dissociation technique passing the tissue through sieves and then separating the resultant cell suspension with Ficoll - sucrose density gradients. Since this initial work, many other authors have reported methods for the separation of neurons and glia, (or more specifically astrocytes), using broadly similar techniques, e.g. Flangas and Bowman (1968); Blomstrand and Hamberger (1969); Sellinger et al (1971); Chao and Rumsby (1977); Farooq and Norton (1978). These methods have been reviewed by Henn (1980) and all follow the same basic pattern. First, the tissue is minced and then may or may not be treated with enzyme(s) (such as trypsin), to weaken cell - cell bonds. The chopped tissue is then mechanically disaggregated by passing it through steel and/or nylon meshes to produce a cell suspension. The buffer used for the cell separation varies between authors, e.g. Rose (1965) used a buffer with a high $[K^+]$ to maintain intracellular K^+ levels and to reduce cell damage (Sinha and Rose 1971), whereas that of Blomstrand and Hamberger (1969) contained lower concentrations of K^+. Separation of the cell suspension into various fractions is then achieved by differential and/or density gradient centrifugation using sucrose (Korey, 1957), sucrose-Ficoll (Rose 1965), Ficoll (Flangas and Bowman, 1968) or Percoll (Trachtenberg and Packey, 1984; Chatterjee and Sarkar, 1984). The purity of the cell enriched fractions produced by these methods was usually established on morphological grounds, although estimates of glial-specific proteins such as glutamine synthetase (Albrecht et al, 1982), carbonic anhydrase (Snyder et al, 1983) or glial fibrillary acidic protein, (Grisar et al, 1983) have also been used to establish the enrichment of a fraction with a particular cell type. Farooq and Norton (1978) reported a method for the separation of rat forebrain astrocytes which yields cells in the highest purity

(70%) and level of structural integrity, with a good yield (2 mg astrocyte protein per 60 day old rat forebrains). Their method uses a dissaggregation technique of aspirating trypsin-treated rat forebrains through glass nozzles and filtration under gravity of the resultant suspension through a comparatively wide (420 μm) nylon mesh. There is only one step in the disaggregation process of Farooq and Norton (1978) where the tissue is forced through the mesh and this produces a fraction enriched in cells having a small nucleus, little perinuclear cytoplasm and extensive long delicate processes. These cells were identified as protoplasmic and fibrous astrocytes under the light microscope, however the more rigorous techniques of cell identification by cell specific antibodies have not commonly been used to check the homogeneity of cell types in fractions obtained by bulk separation. With hindsight, the precise purity of bulk separated cell fractions must be considered to some extent to be unproven.

New methods of cell separation have been suggested which are more rapid (to reduce the inevitable cell damage involved in tissue disaggregation), of a higher yield and also more specific (see Varon and Manthorpe, 1980). The most promising methods use cell type-specific surface antibodies, for example to label with a fluorochrome linked antibody, cells which are then separated by fluorescence activated cell sorting (FACS). This is a process whereby each individual cell in a suspension pre-treated with a fluorochrome linked antibody is then automatically examined and then selected for the presence or absence of fluorescent label by the measurement of their laser stimulated fluorescence emissions (Bonner et al, 1972). Alternatively, antibodies may be used to remove particular cells directly from a mixed cell suspension by

immunoaffinity methods (Varon and Manthorpe, 1980), which involve the binding of a cell type specific surface antibody to a substrate to act as a bond between the selected cell and the substrate. These techniques have been limited primarily to use in neonatal brain tissue culture. For example the oligodendrocyte surface specific antibody 04 linked to magnetic microspheres has been used to select for a population of oligodendrocytes from a neonatal rat cerebellar suspension (Meier et al, 1982; Meier and Schachner, 1982). Unfortunately the lack of an antibody specific to the astrocyte cell surface has restricted the use of these methods.

It would appear that the separation of astrocytes from the adult rat cortex, theoretically the best model for differentiated astrocytes, is best achieved by the use of the bulk separation technique of Farooq and Norton, (1978). This chapter will therefore describe experiments to examine the possible use of this method, or modifications thereof to produce a preparation enriched in viable and identifiable astrocytes which might then be used in further experiments to determine the effect of various and putative neuronal signals on the biochemistry of astrocytes. The enrichment of the astrocyte fractions was determined not only by light microscopy but also by immunofluorescence studies using an antiserum to GFAP. The viability of the cells was judged by trypan blue exclusion and two nucleic acid dyes, ethidium bromide and acridine orange. As the yield and viability of the bulk-prepared cells were unsatisfcatory, efforts were made to raise an antiserum to membranes prepared from the astrocyte enriched fraction derived from a modified Farooq and Norton (1978) technique and the results from this work are also shown. The aim of these latter experiments was to produce an astrocyte surface specific antibody which could be used in the separation of

Astrocytes from the adult rat cortex

Methods

The method chosen to form the basis of a technique for the bulk separation of astrocytes from the adult rat cortex was that of Farooq and Norton (1978). After initial studies using the original procedure, various modifications of this method were made with the aim of determining the optimal separation conditions for the preparation of intact and apparently viable cells (see Cell Viability). The modifications were made as a result of numerous control experiments including:-

a) a comparison of the use of siliconised and non-siliconised glassware during tissue dissaggregation.

b) the reduction of the differential density gradient centrifugation speed.

c) a comparison of vortexing and manual dispersion of the precipitate from S1 (see Farooq and Norton, 1978).

d) replacement of the final aspiration of non-disrupted tissue by a process of gently forcing the tissue through nylon mesh covering the end of a disposable syringe.

e) studies on the optimal method of removing contaminating Ficoll from astrocyte-enriched fractions. Preliminary studies such as these resulted in the use of two methods for the separation of astrocytes.

Method 1

This was very similar to the original Farooq and Norton (1978) method but differed primarily in the elimination of part of the differential density gradients and in that it was performed both with and without trypsin. The original procedure employed a 32% Ficoll layer below the interface at which astrocytes were found (22% and 28% Ficoll) and this was eliminated.

Six 50 day old rats were killed by decapitation, the brains removed and cleaned of cerebellum, midbrain and brain stem. The remaining tissue was chopped into 16-20 slices per brain using a scalpel and transferred to a tube containing 9 mls of disaggregation buffer (8% w/v glucose, 5% w/v fructose and 2% w/v Ficoll, in 10 mM KH_2PO_4-NaOH buffer, pH 6.0) with or without 0.1% acetylated trypsin (Sigma, Type V-S). The tissue was incubated at 37°C for 90 minutes after which time the buffer was removed and 9 mls of 0.1% soybean trypsin inhibitor (Sigma, Type 1-S) in disaggregation buffer was added if trypsin had been used. After five minutes the inhibitor solution was discarded and the slices washed in ice-cold disaggregation buffer four or five

times.

All subsequent steps were carried out at 4°C. Disaggregation was achieved by sucking the slices through a cut off glass pipette of nozzle diameter 2.2 - 2.4 mm and length 3.4 - 3.5 mm, attached by a 2 cm length of tubing to a 250 ml side-arm conical flask containing 20 mls of disaggregation buffer. The flask had been siliconised with Repelcote (Hopkins and Williams Ltd.), as had the glass pipette and tubing. A slight negative pressure to draw the tissue through the glass nozzle was provided by a water pump connected through the neck of the flask.

After the material had been drawn through the nozzle, the residue in the pipette and side-arm were flushed through with 10 mls of disaggregation buffer and the partly dispersed tissue was filtered over a 425 μm nylon mesh (Stanier Ltd., Manchester) into a beaker to give a filtrate F_1. Any material remaining on the screen was then re-drawn through the nozzle into fresh buffer and filtered to give a filtrate F_2. This procedure was repeated to give two more filtrates F_3 and F_4. The material remaining on the mesh after the final filtering was washed into 10 ml of disaggregation buffer and transferred into a 10 ml disposable syringe whose end had been replaced with a covering of 425 μm mesh. The suspension was then gently pushed through the nylon mesh into 10 ml of disaggregation buffer to give a filtrate F_5. All five filtrates were then combined and allowed to settle for 15-20 minutes. The supernatant (S_1) was retained and the precipitate resuspended in 20 mls of disaggregation buffer and shaken by hand. This was left to settle for 15 minutes, whereupon the supernatant was added to S_1 and the precipitate discarded. The combined

suspensions were concentrated by centrifugation at 2,000 r.p.m. (720 g) for 15 minutes. The cell rich pellet thus obtained was suspended in 64 ml of 7% Ficoll in disaggregation buffer and divided into two tubes which were spun at 1,200 r.p.m. (280 g) for 10 minutes to give a pellet P_1. A second pellet P_2 was obtained by re-centrifugation of the supernatant for 2,000 r.p.m. (720 g) for 10 minutes. This also gave a new supernatant which was diluted 1 : 1.125 with disaggregation buffer and recentrifuged at 2,500 r.p.m. (1120 g) for 15 minutes to give the astrocyte enriched pellet P_3. P_2 and P_3 were then resuspended in 34 ml of 7% Ficoll in disaggregation buffer and 15 mls of each were layered into four three step gradients of 10%, 22% and 28% Ficoll in disaggregation buffer. These gradients were centrifuged for 5 minutes at 7,000 r.p.m. (6,400 g) in a Prepspin 75 ultra-centrifuge (MSE Scientific Instruments). Various fractions from these gradients were taken for further analysis. In some experiments the astrocyte enriched fraction P 3/22, i.e. those layers on the P_3 gradients at the 22% and 28% Ficoll interfaces, were transferred to 2 x 50 ml centrifuge tubes to be washed free of contaminating Ficoll. This was performed by the slow, dropwise addition of a modified Kreb's solution to the P 3/22 fractions (NaCl, 110 mM; KCl, 40 mM; $CaCl_2$, 1.2 mM; $MgCl_2.6H_2O$, 1.3 mM; $Na\ H_2\ PO_4.H_2O$, 1.2 mM; glucose, 10 mM; HEPES buffer, 20.0 mM). When the fraction had been diluted 8 - 10 times the dilution rate was increased until 35 mls of buffer had been added. The diluted fraction was then centrifuged at 1,100 r.p.m. (210 g) for 55 minutes. The supernatant was discarded and the pellet resuspended in 1 ml of the modified Kreb's solution.

Method 2

In this variant, the gradient centrifugation step was removed in an attempt to increase cell yield and instead the P_3 pellets were resuspended in 10 - 15 mls of disaggregation buffer and passed through a 1.5 x 1.2 cm diameter column of glass beads (Sigma Type V, 450 - 500 µM; pre-cleaned in nitric acid) made in a plastic funnel whose end was covered with 425 µm mesh. This gave a final suspension PP_3.

Aliquots were taken at various stages during the separation processes and examined under the light microscope or analysed for protein content (Lowry et al., 1951) using bovine serum albumen as standard (BSA; Sigma fraction V). Fractions were also taken for viability estimation using trypan blue and ethidium bromide/ acridine orange, immunofluorescence studies and to raise an antibody to membranes prepared from an astrocyte fraction.

Cell viability

Cells were judged as viable if they excluded trypan blue (0.1%) or fluoresced green after exposure to an equimolar mixture of 1 ppm ethidium bromide and acridine orange, red/orange fluorescent cells being regarded as dead (Pantazis and Kniker, 1979; Jarnagin and Luchsinger, 1980). For fluorescence-examination, cells were viewed under fluorescein and rhodamine optics mounted on a Zeiss photomicroscope II.

GFAP immunofluorescence

In two experiments unwashed P3/22 aliquots were labelled with an antiserum to GFAP (a gift of Prof. M. Raff) using the indirect immunofluorescence technique. Aliquots of cell suspension (approx. 50 - 100 µl) were placed onto 22 x 22 mm glass coverslips coated with gelatin (0.5% gelatin; 0.05% chrome alum) and allowed to settle for 15 minutes. The coverslips were then rinsed in phosphate buffered saline (25 mM NaH_2PO_2/Na_2HPO_4, 0.9% NaCl pH 7.4; PBS). The cells were fixed in acid/alcohol (1% HCl in 70% ethanol) for 5 minutes at -20°C and rinsed again in PBS. 100 µl of anti-GFAP antiserum diluted 1 : 125 in PBS was then added to the coverslips for 30 minutes. In order to wash out any non-bound anti-GFAP, and to block non-specific binding of the second antibody, the coverslips were then rinsed 4 - 5 times in 5% normal lamb's serum (Gibco) in PBS over 10 minutes. The second antibody was then added at a dilution of 1 : 50 in PBS for 30 minutes (goat anti-rabbit-rhodmine conjugate, Miles Biochemicals Ltd.). After this time the cells were rinsed in 5% normal lamb's serum as before. The coverslips were then mounted onto glass slides in glycerol and examined under rhodamine optics using a Zeiss photo-microscope II. Photographs of the labelled cells were taken using Kodak Ektachrome 400 ASA film.

B1 antiserum

Adult cortical astrocyte membranes were prepared by lysing in ice cold distilled water a suspension of material obtained by passing a P3/22 fraction through glass beads as in Method 2 and centrifuging the lysate at 50,000 g in a Sorvall RC-5B ultra-

centrifuge (Du Pont Instruments) for 30 minutes. The membranes were resuspended in PBS and mixed with an equal quantity of Freund's Complete Adjuvant to a final concentration of 200 µg protein/ml. 500 µl of this solution was injected intradermally at 5 sites on the back of a female New Zealand white rabbit (2.5 - 3 kg). Two and five weeks later the animal was boosted with the injection of 400 µg protein of the membrane preparation in Freund's Complete Adjuvant at 10 sites along the animal's back. Blood was taken from the ear vein at 6, 11, 13 and 20 weeks after the initial injection. After allowing the blood to clot overnight at 4°C, the serum was separated by centrifugation at 2,500 r.p.m. (1100 g) for 15 minutes. Immunoglobulins were extracted from the serum by double ammonium sulphate precipitation (0.31 g/ml serum) and centrifugation at 50,000 g for 30 minutes. The final pellet was resuspended in 50 mM sodium phosphate pH 7.4 to a concentration of 5 mg protein/ml and aliquots frozen at -70°C. This antiserum was partly screened by immunodotting, immunoblotting and immunofluorescence.

Immunodotting

Immunodotting, based on Hawkes et al (1982) was carried out as follows. Approximately 2.0 and 0.2 µg protein aliquots of various tissue homogenates in distilled water were dotted onto nitrocellulose paper grids (1 x 1.5 cm, Bio-rad) in duplicate or triplicate and allowed to dry overnight. The grids were washed twice in 1 ml of Tris buffered saline (TBS; 50 mM Trizma base; 200 mM NaCl, pH 7.4) with shaking at room temperature. A solution of 3% BSA (Sigma, fraction V) in TBS was applied for 60 minutes to block non-specific binding sites for protein on the grids and incubated at 4°C overnight after which they were washed

4 times with 1 ml of TBS for 20 minutes each. Blocking of non-specific sites was performed as before and then 1 ml of the second antibody (goat anti-rabbit-horseradish peroxidase conjugate) diluted 1 : 500 in 3% BSA/TBS was added to the grids for 2 hours. Any non-bound antiserum was removed by washing four times with 1 ml TBS each for 20 minutes. Finally, the bound second antibody was visualised by incubating the samples with a solution of 0.06% chloro-1-napthol, 20% methanol, 0.07% hydrogen peroxide in TBS. Incubation was continued until reaction product appeared at the site of the dot, and was then stopped by washing with distilled water.

Immunoblotting

Immunoblotting was performed by transfering proteins from homogenates of rat cerebral cortex, astrocyte and meningeal cell cultures (see Chapter 3 for culture methods) separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) onto nitrocellulose paper as described by Towbin et at (1979). SDS-PAGE separation of proteins was essentially by the methods of Laemmli (1973) and Mahadik et al (1976). Briefly, homogenates in 5 mM Tris HCl pH 8.4 were diluted 1 : 1 with dissociation buffer (SDS, 1.25%; 2-mercaptoethanol, 1%; EDTA, 2 mM; sucrose, 10%; Tris-HCl, 125 mM; pH 6.8) to 1 mg protein/ml and boiled for 5 minutes. 100 µl aliquots and molecular weight markers (Sigma, SDS - 6H) were then run on a 10% polyacrylamide gel at 40 mA for 4 - 5 hours using bromophenol blue to show the gel front. The gel-separated proteins were then transferred to nitrocellulose paper using a Biorad immunoblotting apparatus with a current of 1.8 A, 60 mV for two hours. The immunoblot was then divided into vertical strips containing either molecular weight standard proteins or

homogenate proteins. Strips were strained for proteins with amido black (0.1% w/v amido black, 10% acetic acid, 25% iso-propyl-alcohol); strips with separated homogenate proteins were treated as described for immunodots using either B1 antiserum (1 : 50) or rabbit pre-immune serum (1 : 50) in 3% BSA/TBS.

Immunofluorescence studies

The intracellular and extracellular distributions of the antigen(s) bound by B1 were examined by immunofluorescence on astrocyte enriched cultures (Chapter 3), cerebellar granule cell cultures (prepared by Dr. B. Pearce by the method of Dutton et al, 1981) and growth cones from 5 day old rat forebrain (prepared by Dr. P. Gordon-Weeks as in Gordon-Weeks and Lockerbie, 1984). The method used for immunofluorescence was essentially as described here and is shown in Chapter 3.

"Panning" for $B1^+$ cells

Two attempts were made to use the panning technique of Wysocki and Sato (1978) to separate $B1^+$ cells from suspensions using the second modified method of Farooq and Norton (1978).

B1 antiserum was diluted 1 : 100 or 1 : 1000 (50 and 5 µg protein/ml) in 50 mM Tris-HCl pH 9.5 and 5 mls of each dilution were added to 6 x 50 mm diameter non-tissue culture grade plastic petri dishes (Sterilin) for 40 minutes at room temperature. The dishes were then washed four times with 5 mls of PBS and once in 3 mls of 1% foetal calf serum (Flow; FCS) in PBS. Cell suspensions (3 mls) from the pooled filtrates (F_1 - F_5), cell rich pellet (non

cell enriched) resuspended in 5% FCS/PBS and astrocyte enriched suspension (PP_3) were then added to the antibody coated dishes in duplicate. After 40 minutes at 4°C or room temperature, the dishes were gently swirled, then left for a further 30 minutes before being examined under the light microscope, washed four times with 1% FCS/PBS and further examined.

Results

Bulk cell separation

When fractions prepared by the original Farooq and Norton (1978) method, and the two variations previously described were examined under the light microscope, cells of a morphology previously described as astrocytic (Farooq and Norton, 1978; Albrecht et al, 1982) could be observed in the P3/22 and PP_3 fractions. These cells were characterised by the possession of a small bean-like nucleus with little cytoplasm and extensive fine processes. They could be observed in the P3/22 fraction with or without pre-treatment with trypsin although not in P3/22 fractions prepared using the higher speed gradient centrifugation (8,500 g) of Farooq and Norton (1978). The elimination of the lowest step in the density gradient centrifugation process (32% Ficoll in disaggregation buffer) did not have any apparent effect on the yield and composition of the P3/22 fraction and presumed astrocytes in the P3/22 and PP_3 fractions could be washed free of Ficoll without further noticeable cell damage. Photomicrographs of cells variously prepared are shown in Figs 2.1.

Additionally, cells of the above morphology are seen to label

Table 2.1 Protein yield of cell separation fractions

Fraction	Recovery (%)
P_1	2.6 ± 0.3 (16)
P_2	1.2 ± 0.3 (15)
P_3	0.7 ± 0.1 (14)
$P2/_{22}$	0.4 ± 0.1 (6)
$P3/_{22}$	0.2 ± 0.1 (8)
PP_3	0.2 ± 0.1 (6)

Legend

The results shown here are the mean ± SEM of the recovery (expressed as % starting material, assuming 100mg protein per pair of rat cortices) in various fractions pooled from all three methods with or without trypsin. All PP_3 determinations were made on cells prepared without trypsin. All aliquots were washed by centrifugation with 10-15 vols of PBS (750g, 15 mins) prior to protein estimation. Numbers in brackets refer to the number of experiments

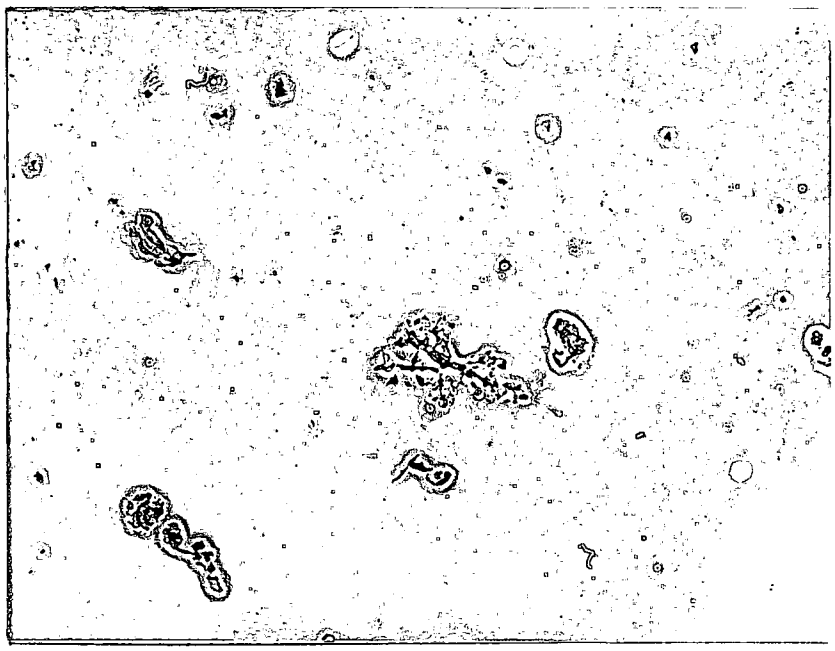
Fig. 2.1

Micrographs of cells separated from the adult rat cortex

a) A typical cell of the type seen in "astrocyte" fractions, in this case the $P^{3}/_{22}$ fraction, x 650.

b) A cell from the PP_3 fraction, x 1500.

a



b

with antiserum to GFAP (Fig 2.2). However, the yield and composition of the "astrocyte enriched" fractions varied greatly. Table 2.1 shows the protein yield at various stages during the cell separation processes. The yield in the two "astrocyte enriched" fractions is particularly low, P3/22 = 1.2 ± 0.3 mg, PP_3 = 1.2 ± 0.4 mg from 6 x 40 day rat cortices. If it is assumed that one rat brain constitues 100 mg of cortical protein this represents a recovery of approximately 0.2%. Furthermore, frequently nothing of a well defined cellular shape could be determined in the P3/22 or PP_3 fractions and debris always predominated. None of the cells observed excluded trypan blue and most fluoresced predominantly red when exposed to acridine orange/ethidium bromide, indicating cell damage. Moreover, presumed capillaries were often observed in the P3/22 fraction (Fig 2.3). The PP_3 suspension however did not contain obvious capillary contamination, and neuronal cell bodies, (identified as phase bright cells with a pear shaped perikaryon and few processes) were rarely seen in the P3/22 and PP_3 fractions.

B1 antiserum

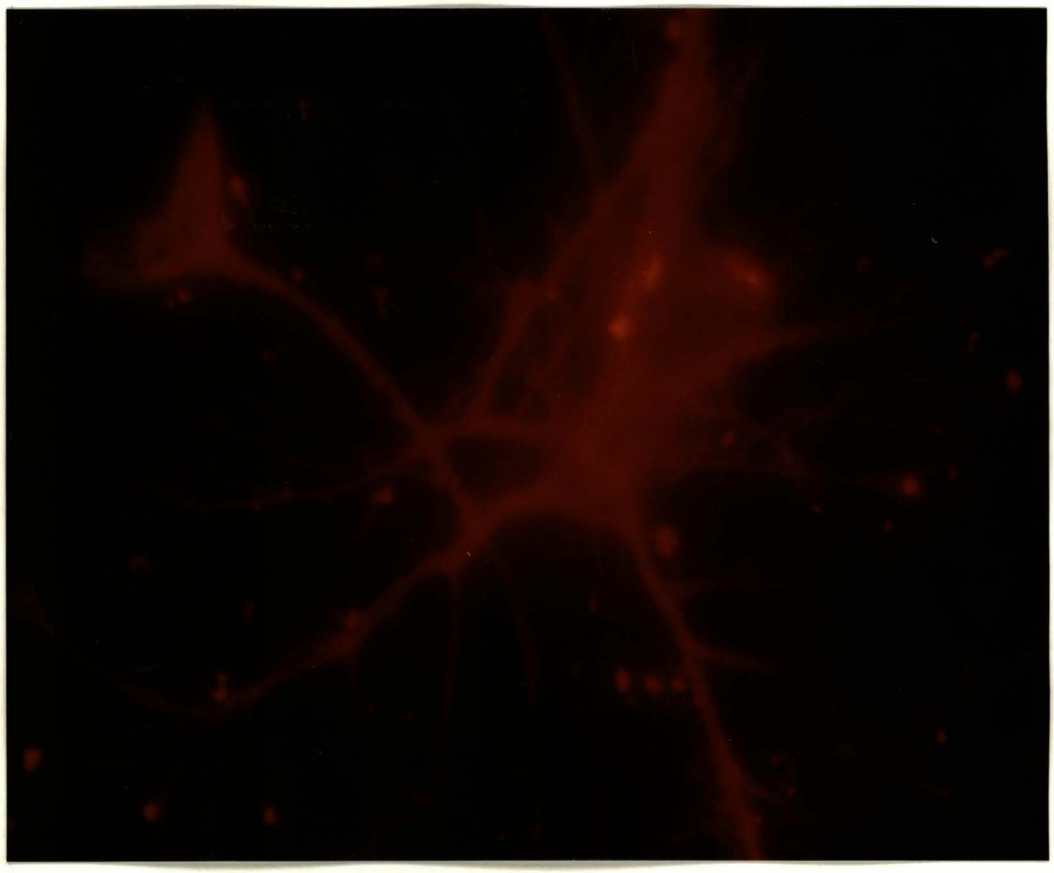
Attempts to raise an astrocyte cell-surface specific antiserum resulted in an antiserum B1 which bound readily to homogenates of rat brain, Fig 2.4a. However, the antigen(s) recognised by B1 in rat brain is/are also present in peripheral tissues such as liver, kidney, heart and lung, Fig 2.4b. Immunoblotting SDS-PAGE of 50 day rat cortex and astrocyte and meningeal cell cultures with B1 produced a labelled band of protein of approximate molecular weight 42,000 Daltons Fig 2.4c. B1 also binds to purified actin (from chick brain, a gift from P. Tilson) Fig 2.4b. Additionally in immuno-

Fig. 2.2

Immunofluorescence micrographs of cells separated from the adult rat cortex

a) and b) GFAP immunofluorescence micrographs of cells present in the $P^{3}/_{22}$ fraction, x 3000. Control coverslips incubated with PBS instead of anti-GFAP antiserum showed no fluorescence. Cells in micrograph (a) and (b) are viewed through gelatin/chrome alum which produces a hazy image.

a



b

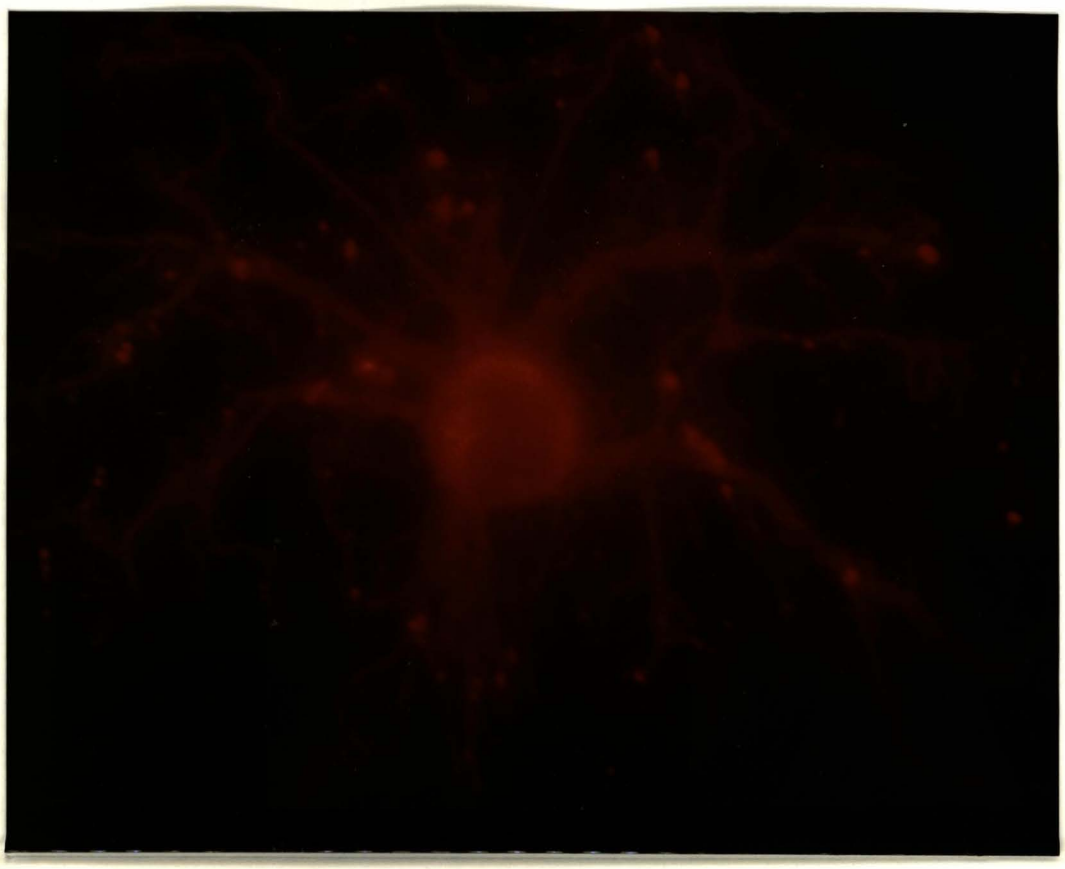


Fig. 2.3

Micrograph of a capillary

A presumed capillary observed in the $P^{3}/_{22}$ fraction, x 400.

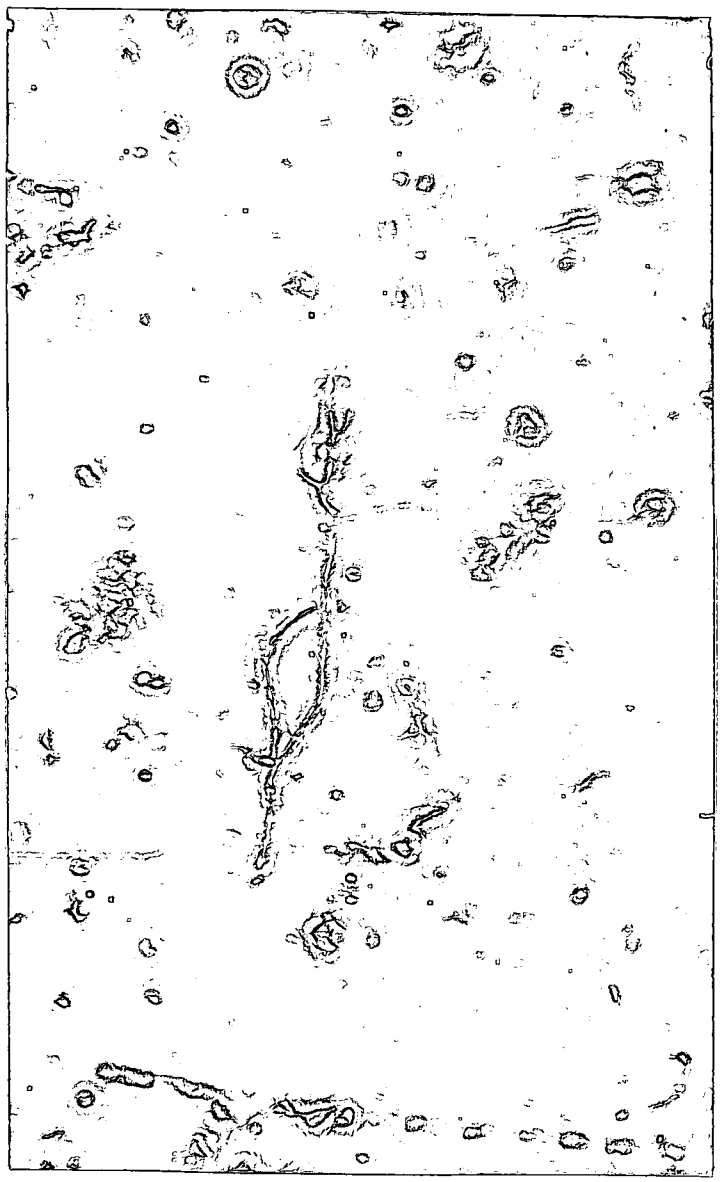


Fig. 2.4

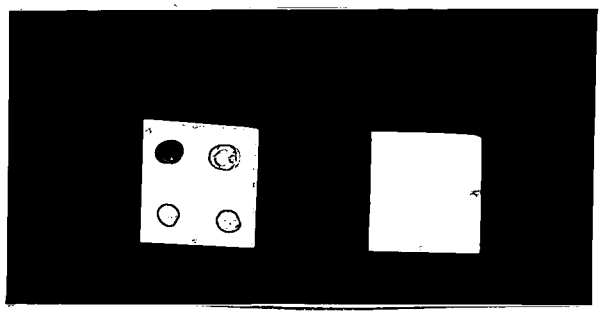
Characterisation of B1 by immunohistochemistry

a) Immunodots of duplicate 2.0 and 0.2 µg aliquots using B1 (left) and pre-immune rabbit serum (right)

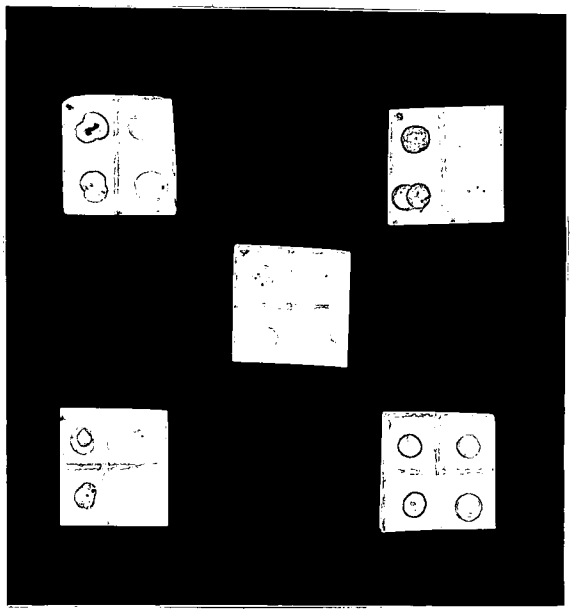
b) Immunodots of duplicate 2.0 and 0.2 µg aliquots of actin and from left to right, top to bottom, heart, lung, actin, liver and kidney, using B1

c) Immunoblot of homogenates of astrocyte-enriched (A) and meningeal cell (F) cultures using B1 (+) and pre-immune serum (-)

a



b



c

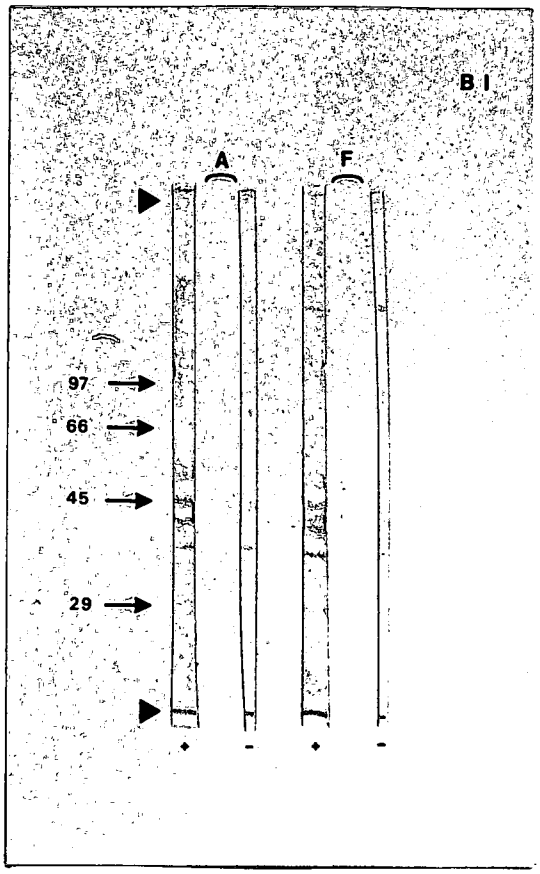
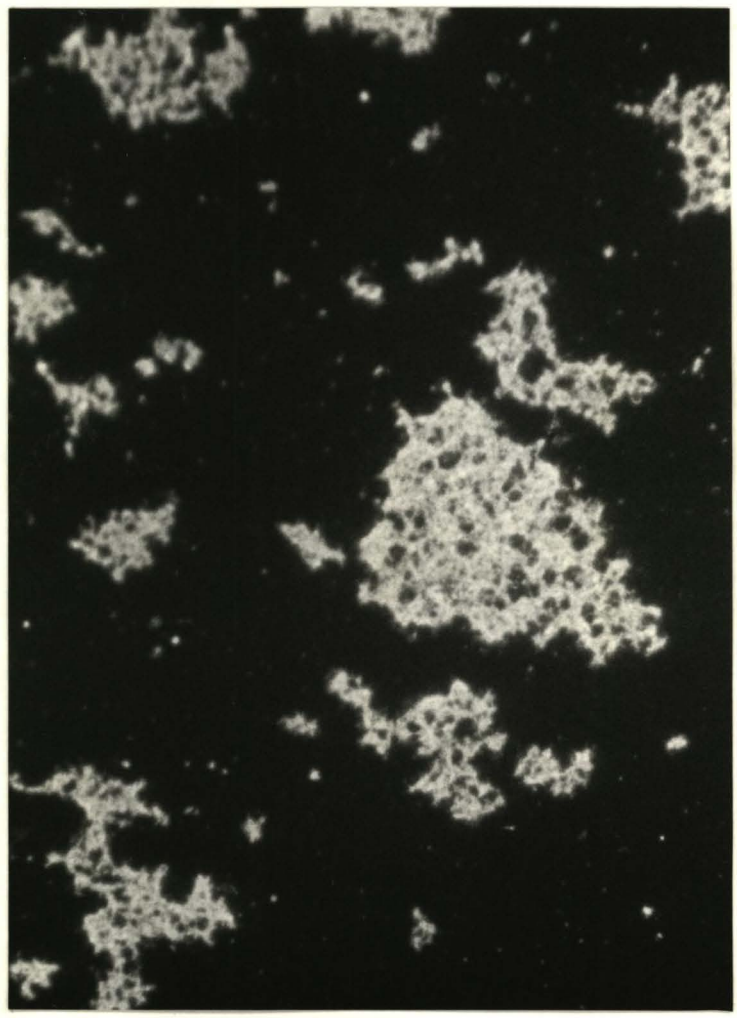


Fig. 2.5

Immunofluorescence micrograph of growth cones from neonatal rat cortex

Immunofluorescence micrograph of B1 internally labelling growth cone aggregates in vitro, x 1800.



fluorescence studies of astrocyte enriched cultures, B1 labels intracellular fibres of all cells present but only at low dilutions < 1 : 5. In addition the antiserum labels growth cones markedly (Fig. 2.5). The antiserum labels the surfaces of all cells present in astrocyte-enriched cultures and in cerebellar granule cell cenriched cultures with a speckled, dot-like appearance.

The use of B1 in panning experiments resulted in almost no cells or debris from any of the suspension becoming attached to the substrate before washing in 1% FCS/PBS abd nothing remained attached after washing.

Discussion

The results have been presented from experiments designed to modify a previously existing technique (Farooq and Norton, 1978) to produce reproducibly a fraction enriched in viable adult rat cortical astrocytes free from contaminating cell types. Previous studies using this method or its earlier version (Norton and Poduslo, 1970) or modifications of these techniques (Albrecht et al, 1982) suggested that a good yield of well preserved astrocytes could be obtained. The cells observed in such fractions have generally been identified as astrocytes on light microscope morphological analyses alone however. Electron microscope examination of cells produced by these methods is reportedly hindered by the marked tendency of astrocytes to swell and burst during processing for examination (see Hannuniemi et al, 1980) and immunocytochemical analyses of the fractions have not been performed.

The yield stated by Farooq and Norton (1978) for the P3/22 fraction was 12 mg per six 60 day old rats, although this was from the entire forebrain. Indeed their method has formed the basis of a number of biochemical studies (Hannuniemi et al, 1980; Hannuneimi and Oja, 1981; Snyder et al, 1983; Snyder and Wilson, 1983). Hannuniemi et al (1981) used three week old rat cortices and a modified version of the technique of Norton and Poduslo (1970) to prepare astrocytes with a yield of 10 mg protein per g wet weight cortex. However, the yield reported here of astrocytes, (identified as $GFAP^+$ cells by immunofluorescence), from the rat cerebral cotex is very low ($\bar{x}$ = 1.2 mg from six rats, or approximately 0.2 mg per g wet weight cortex assuming two cortices/g wet weight). This is so when using the original unmodified technique or a slightly modified version which reduces the number of gradient layers in the density gradient step.

In an attempt to increase the yield of astrocytes and to remove the capillary contamination noted here in the P_3 fraction and also reported by Farooq and Norton (1978), a second modification was used whereby the astrocyte enriched pellet P_3 was resuspended and filtered through a column of glass beads. This principle has been used previously to remove capillaries from a cell suspension (Rose and Sinha, 1970). Whilst the removal of capillaries was successful, the yield was again low, being approximately 1.2 mg protein for 6 rat brains.

The reason for such low yields obtained here is unclear. Notably, Chatterjee and Sarkar (1984) have stated that protoplasmic astrocytes, the predominant cell type in the cerebral cortex, are very susceptible to damage by a proteolytic enzyme (trypsin).

This may account for the relatively low yield and high amounts of debris in astrocyte fractions apparent here when using trypsin treated cerebral cortex. Farooq and Norton (1978), whilst also using trypsin, utilised the entire forebrain as starting material which contains both protoplasmic and fibrous astrocytes. Also, although Hannuniemi et al (1980) prepared astrocytes from the cerebral cortex, they used a reduced (45 minutes) incubation with trypsin. A marked sensitivity to proteolytic enzymes may also account for the comparatively meagre yield of astrocytes observed even when the cortices were not exposed to added trypsin. The non-trypsinised, chopped tissue, which was also incubated at 37°C for 90 minutes as it proved easier to disaggregate than if no incubation was used, may release a variety of proteases from damaged cells which could have had deleterious effects on the intact astrocytes.

Certainly the cells which are produced by the original method and its modifications are badly damaged and non-viable as judged by trypan blue exclusion and acridine orange/ethidium bromide staining irrespective of whether the cortices had been pre-treated with trypsin. Moreover, intact cells were by no means always present in the "astrocyte" fractions. It has been reported previously that cells prepared by the Farooq and Norton (1978) method or similar are badly damaged and may lose cytoplasmic constituents such as the astrocyte-specific enzyme glutamine synthetase (Hannuniemi et al, 1980; Albrecht et al, 1982). This damage is apparent before any attempts are made to remove the non-physiological disaggregation buffer containing Ficoll and/or sucrose. Hannuniemi et al (1980) have suggested that this must be performed by very slowly diluting the disaggregation buffer by

dialysis with a physiological buffer. The astrocytes prepared here could be washed apparently free of Ficoll and still remain intact but only by a very slow dilution with buffer added manually and pelleting by centrifugation.

Due to the low yield and poor structural integrity of the isolated astrocytes, alternative methods for producing enriched cell populations were examined. One of these was the possibility of raising an astrocyte specific cell surface antibody which could be used to separate astrocytes by an immunoaffinity method. Thus a P3/22 astrocyte fraction (prepared without trypsin) was passed through glass beads to remove capillaries and then membranes prepared from this fraction were injected into a rabbit. The serum resulting from this innoculation contained antibodies to determinant(s) in the adult cortex as shown by immunodotting (Fig 2.6). However, the determinant(s) were also found in homogenates prepared from other tissues such as kidney, lung, heart and liver. The immunoblotting study showed that the anti-serum bound most avidly to a protein of molecular weight 42,000 Daltons and immunofluorescence showed that it bound to intra-cellular filaments in astrocyte or meningeal cell cultures although only at low dilutions (1 : 5 or less).

The resulting serum (B1) did indeed label the surfaces of astrocytes in culture at a comparatively high dilution (1 : 50). However, all the cells in astrocyte cultures were labelled, including cells believed to be derived from meninges (see Chapter 3). Furthermore, cerebellar granule cells in vitro were also labelled with B1. When immunoblotting studies were performed (figs. 2.4a,b) it was clear that whilst B1 bound to determinant(s) in the adult cortex, it also bound to determinant(s) in the kidney, lung, heart and liver. Immunoblotting studies were therefore performed to examine further the nature of the antigen(s) labelled by B1. These studies (fig. 2.4c) showed that B1 bound most avidly to a 42,000 Dalton protein from 10% polyacrylamide gels of cell culture homogenates. This result suggested that B1 bound to actin. Indeed B1 bound to actin immunodots and also to actin rich growth cones in immunofluorescence studies (fig. 2.5). B1 also labelled intracellular fibres in cell cultures as shown by immunofluorescence although at lower dilutions than that seen for surface labelling (1 : 5 compared to 1 : 50). It is unlikely therefore that actin-immunoreactivity accounts for the labelling by B1 of cell surfaces. In one B1 immunoblot of a 10% polyacrylamide gel of astrocyte culture homogenates, a darkly labelling band was observed at the top of the blot suggesting that the antiserum recognised a high molecular weight determinant (> 200,000 Daltons). This determinant may be that which is present on the surfaces of cells in vitro. However, as the surface antigen was not cell-type specific and therefore unlikely to be suitable for use in cell separation, further investigation was not attempted.

In spite of the lack of specificity of the antiserum to cells in vitro it was possible that the antigen might become restricted to astrocytes at a later developmental stage. Therefore attempts to use the B1 antiserum to separate adult cortical astrocytes by the "panning" method of Wysocki and Sato (1978) were continued. This method was originally developed for the separation of B and T lymphocytes and involves the binding of a cell specific antibody to non-tissue culture grade dishes and allowing cells expressing the corresponding antigen to bind to the antibody, thus providing a means for separation. However, this technique did not result in the binding of any cell or cell fragment from a variety of cell suspensions prepared from the adult rat cortex to the B1 coated substrate. The reason for this is not clear but might reflect a lack of binding of B1 to the substrate or alternatively that the B1 antiserum cross-reacts with a determinant present on the surface of cells in vitro which is not expressed in the adult cortex.

In summary therefore, an enriched fraction containing a high yield of predominantly viable, identifiable astrocytes could not be produced by applications of the bulk separation technique of Farooq and Norton (1978) to the adult rat cerebral cortex, although cells identified as astrocytes by anti-GFAP immuno-fluorescence could be observed. Attempts to raise an antiserum specific to the surface of astrocytes which could provide the basis for an alternative immunoaffinity separation method

were also unsuccessful. In the light of these results an alternative model preparation for astrocytes in vivo was considered, namely the in vitro cultivation of neonatal rat cortical tissue to yield astrocyte-enriched primary cultures.

CHAPTER 3

Astrocytes in primary culture

Introduction

The previous chapter described attempts to produce an enriched preparation of viable astrocytes from the cerebral cortex of adult rats by modifications of a previously published method. These attempts resulted in a preparation which did not always contain identifiable astrocytes and was never free of substantial amounts of contaminating material as judged by light microscopy. Cells, tentatively identified as astrocytes, were also seemingly damaged. It was therefore decided to examine the possibility of using one of two alternative techniques by which a preparation, measurably enriched in rat cortical astrocytes, might be obtained. These alternatives were the use of clonal cell lines such as C6 glioma or of primary cultures of the neonatal rat cortex. The former technique was not chosen for reasons outlined in Chapter 1. Thus primary cultures of neonatal rat cortex were evaluated for their degree of enrichment with astrocytes and the presence of identifiable contaminating cell types.

The first tissue culture of CNS was performed in 1907 by Harrison who cultured embryonic frog spinal cord in lymph clots to examine the outgrowth of nerve fibres. Between the two World Wars most CNS culture work was performed using glioma cells e.g. Buckley (1929), see Ponten and MacIntyre (1968) for references. In the 1950s, explant culture of neural tissue was used to examine a

variety of properties of CNS cells e.g. pulsatile activity (Pomerat, 1951) or electrophysiological studies (Hild et al, 1958). Nakai in 1956 reported the first dissociated cell cultures of nervous tissue, using spinal cord dorsal root ganglia, and dissociated culture techniques were used by Shein (1965) to prepare cultures of human foetal brain astrocytes.

More recently, most studies of astrocytes in vitro have used monolayer cultures of glioma cells, usually C6 glioma, or primary cultures from perinatal rodent or chick brain (Varon and Raiborn, 1969; Booher and Sensenbrenner, 1972). Explant cultures of rat brain stem and spinal cord are still in use, particularly in electrophysiological studies (e.g. Hosli et al, 1984), and the growth of astrocytes from adult brain has been reported (Gilden et al, 1976; Singh and van Alstyne, 1978; Lindsay et al, 1982; Vernadakis et al , 1984). In order to produce dividing astrocytes from adult brain, the induction of reactive gliosis in vivo before tissue dissociation is usually considered essential. Singh and van Alstyne (1978) and Lindsay et al (1982) used, respectively, pretreatment with intracerebral injections of the neurotoxin kainic acid, and surgical lesioning, both at least five days prior to culture of the resultant reactive tissue. The need for induction of a glial reaction before adult astrocytes will divide in vitro is also reflected in the early work of Ponten and MacIntyre (1968). They, whilst describing the growth of normal as well as neoplastic astrocytes from (presumably) adult human brains, used biopsy tissue for their 'normal' glial cultures from wound debridement or from areas around haematoma or aneurysms. In contrast, the report of Vernadakis et al (1984) suggests that astrocytes can be grown from adult mouse brain without prior insult if the dissociated

cells are left for 9-10 days to allow adherence to the culture substrate. Also Gilden et al (1976) report that dividing astrocytes in vitro may be obtained from explants of adult human white matter. The use of in vitro adult astrocytes however is very limited and as previously stated most primary cultures of rodent astrocytes use neonatal brain as starting material.

In general, most methods for the preparation of astrocyte enriched primary cultures from neonatal rodent brain follow the same basic principles. First, the brains from neonatal pups are removed and cleaned of surrounding meninges. Neonatal pups are used as the bulk of astrocyte division occurs at this stage in vivo (see e.g. Parnavelas, 1983). Cultures of forebrain astrocytes are usually derived from pups at 1 or 2 days post-natal and of cerebellar astrocytes from 8 days postnatal (Cohen et al, 1979; Currie and Dutton, 1980). After removal of meningeal tissue, further dissection may then take place to separate particular areas (e.g. Hansson, 1984) or to mince the brain prior to enzyme treatment. Enzymes, usually trypsin, may or may not be used prior to disaggregation of the tissue by gently forcing through nylon mesh (Booher and Sensenbrenner, 1972; McCarthy and de Vellis, 1978) or trituration in glass pipettes (Cohen et al, 1979) or syringes with needles of one diameter (Pettmann et al, 1981) or two needles of decreasing diameter (Chapman and Rumsby, 1982). The resulting cell suspension is then pelleted by centrifugation, diluted in culture medium and seeded onto culture dishes or flasks which may be coated with poly-lysine, poly-ornithine, collagen or left untreated. The cells are grown to confluence at 2-3 weeks, with medium changes every 2 - 4 days.

Often the cAMP analogue dibutyryl cyclic AMP (dBcAMP) or brain extracts are added to the culture medium during the last week of growth. This is reported to produce a change from the flattened polygonal astrocytes seen after two weeks in vitro to multi-processed cells, described as a 'differentiation' of the cultured astrocytes from immature astroblasts to fibrous astrocytes (Sensenbrenner et al, 1972; Lim et al, 1973). dBcAMP treated cells express a number of astrocyte specific markers e.g. GFAP (Bock et al, 1975), S-100 protein (Lim et al, 1977), and glial filaments as seen by electron microscopy (Moonen et al, 1976). There is however a current debate as to whether flattened polygonal astrocytes differentiated by dBcAMP are fibrous astrocytes or reactive astrocytes (Fedoroff et al, 1984).

There have been a number of variations from the basic methods of astrocyte culture preparation described previously, designed to enrich further the cultures with astrocytes. McCarthy and de Vellis (1980) used mechanical shaking to remove oligodendrocytes from the surface of astrocyte cultures. This technique was also used by Rougon et al (1983) in conjunction with the method of Cohen et al (1979) and a period (48 hours) of exposure to the mitotic inhibitor cytosine arabinoside to kill rapidly dividing cells which were presumed to be fibroblasts. After treatment with cytosine arabinoside the cells were removed from the tissue culture flasks and replated onto poly lysine coated glass coverslips for immunofluorescence studies.

In general most studies requiring bulk preparation of astrocyte cultures have used either glioma cell lines or the comparatively simple methods of primary culture i.e. those of Booher and Sensenbrenner (1972); McCarthy and de Vellis (1980),

Kimelberg et al (1978) or slight variations therefrom, rather than more complex methods (e.g. Rougon et al, 1983). This latter technique, and those of Cohen et al (1979) and Fedoroff et al (1977), have been used for studies of glial cell development in vitro (e.g. Levi et al, 1983; Wilkin et al, 1983; Fedoroff, 1984) or for experiments where large numbers of cultures or a lot of 'astrocyte' material are not necessary (e.g. Pearce et al, 1981; Rougon et al, 1983)

Early morphological and immunohistochemical studies using antibodies to the astrocyte specific protein GFAP, or electron-microscopy (Bock et al, 1975; 1977; McCarthy and de Vellis, 1980) suggested that astrocyte cultures produced by the methods of Booher and Sensenbrenner (1972) and McCarthy and de Vellis (1980) were highly enriched in astrocytes. However the immunocytochemical, histochemical and morphological studies of Raff et al (1979), Steig et al (1980) and Hansson et al (1980; 1982) have shown that newborn rat forebrain primary cultures prepared using the methods of Cohen et al (1979), Kimelberg et al (1978), and Booher and Sensenbrenner (1972) respectively may contain a variety of cells other than astrocytes. These include oligodendrocytes, mesenchymal cells, endothelial cells, macrophages and ependymal cells. These contaminants may constitute as much as 20 - 30 % of cells present in astrocyte cultures. In particular cells which express fibronectin (FN) at their surfaces are found in astrocyte cultures (Raff et al, 1979; Steig et al, 1980). FN is a large glycoprotein (220 kilo daltons) involved in a variety of cellular functions such as migration and adhesion (Hynes & Yamada, 1982) but is not found on astrocytes in vivo (Schachner et al, 1978) or in vitro (Raff et al, 1979; Steig et al, 1980). However,

Kimelberg and Pelton (1983) and Kimelberg (1983) claim that careful removal of the meninges, commonly regarded as a source of FN^+ cells in astrocyte cultures, can result in 95% pure astrocyte cultures. This figure is in agreement with the figure quoted by Hertz et al (1982) using a culture method similar to that of Booher and Sensenbrenner (1972). These results show the importance of carefully characterising a neural cell culture system.

There is a growing range of antibodies to cell specific molecules (markers) that are useful in determining the type of cells present in neural cell culture systems. The term 'antibody' is used here to cover both monoclonal antibodies and polyclonal antisera. Most of those antibodies relevant to this current study are reviewed by Mirsky (1982). In addition a range of monoclonal antibodies to the cerebellum have been raised and used to examine cerebellar development (Schachner, 1982). Other cell markers to which antibodies have been raised and used to characterise cell cultures and/or to investigate cell lineages include the intermediate filament proteins vimentin (Bignami et al, 1982) and neurofilament protein (Wood and Anderton, 1981); glutamine synthetase (Norenberg and Martinez-Hernandez 1979; Hallermayer et al, 1981); non-neuronal enolase, a glycolytic enzyme specific to astrocytes in the cerellum (Ghandour et al, 1981a); S-100 protein, an acidic Ca^{2+} binding protein found predominantly in astrocytes (Ludwin et al, 1976; Ghandour et al, 1981b; Michetti et al, 1983); 2' , 3' - cyclic nucleotide - 3' phosphohydrolase and myelin basic protein, two myelin associated proteins found in conjunction with oligodendrocytes (Sternberger et al, 1978; McMorris et al, 1984); and ganglioside GQ_{1c}, A_2 B_5

antibody (Eisenbarth et al, 1979; Kasai and Yu, 1983).

This chapter will describe a method for the production of an astrocyte enriched (approximately 85%) culture preparation characterised by the use of an assay for glutamine synthetase and of several antibodies to cell specific markers. Also attempts to enrich further the cultures with astrocytes by three methods are described and discussed with respect to their possible use in routine preparation of large numbers of astrocyte cultures. In addition, a method is described for the preparation of cultures of meningeal cells, which were considered to be the major source of contaminating cells in astrocyte cultures.

Methods

Preparation of cell cultures

Astrocyte enriched cultures were prepared by a modification of previously published methods (Booher and Sensenbrenner, 1972; Pettmann et al, 1981). Neonatal (newborn - 2 days old) rat cortices were cleaned of meninges and subcortical structures under aseptic conditions in PBS and transferred to conical tubes (Sterilin) containing 3 ml of medium, 3 brains per tube. The tissue was then dissociated into a single cell suspension by passage through a 1.5 mm diameter stainless steel cannula. Undisrupted material was allowed to settle under gravity and the cell suspensions (supernatants) were transferred to fresh conical tubes using sterile siliconised pasteur pipettes. Cell debris was removed from the cell suspensions by centrifugation (5 minutes at 180 g) through a 4% (w/v) underlay of bovine serum albumen (Sigma

fraction V). The resultant supernatants were discarded and the cell pellets resuspended in 1 ml of culture medium per tube and pooled. A 20 µl aliquot was then taken from the pooled cell suspension, diluted to 20 mls with Isoton buffer (Coulter) and counted using a Model ZB Coulter counter (Coulter Electronics Ltd.) fitted with a 140 µm orifice tube. The cells were plated onto poly-D-lysine (50 µg/ml) coated 60 mm diameter plastic petri dishes (Falcon), 5 mls medium per dish, or similarly coated 13 mm diameter glass coverslips in a 24 place tissue culture multiwell (Falcon), 0.25 ml medium per coverslip. The seeding density was 780 cells mm^{-2} which represents approximately 2.2 x 10^6 cells per culture dish and 1 x 10^5 cells per 13 mm diameter coverslip. When cells were plated onto coverslips, the cell suspension was allowed to settle onto the coverslip for 30 minutes before the addition of 0.5 ml of medium per coverslip.

Culture medium consisted of Minimal Essential Medium with Earle's salts (Gibco) supplemented with 10% foetal calf serum (Northumbria Biochemicals); 2.5% chick embryo extract (Flow); 2 mM glutamine; 33 mM glucose and 65 µg/ml gentamycin sulphate.

Meningeal cell cultures were prepared by incubating the pooled meninges removed from cortices used for preparation of astrocyte cultures with 2.5 mls of collagenase (2 mg/ml, Sigma Type II) in Earle's Basic Salt Solution, Ca^{2+} and Mg^{2+} free (Gibco) at 37°C for 60 minutes. The tissue was then disrupted by passage through a stainless steel cannula as previously described. The resultant cell suspension was plated onto poly-D-lysine coated 60 mm dishes in normal medium without chick embryo extract. Typically the meninges from 12 animals were used per 20-30 dishes, 5 mls of

medium per dish.

All cell cultures were grown to confluence before use in experiments: 18 -22 days in vitro (d.i.v) for astrocytes, 14 - 18 d.i.v. for meninges. The medium was changed at 6 and 13 d.i.v. for astrocyte cultures and at 6 d.i.v. for meningeal cultures.

Immunofluorescence studies

Experiments to examine the cellular composition of astrocyte enriched cultures using cell specific antibodies and the indirect immunofluorescence technique were carried out on confluent cultures grown on 13 mm coverslips.

The coverslips were placed in a humidified chamber and the cells washed with 2 - 3 mls of PBS. If the antigen being examined was intracellular, then the cells were fixed in acid/alcohol (5% glacial acetic acid/95% ethanol) at -20°C for 10 minutes. Fixed cultures were then washed with PBS as before. Prior to the addition of the first antibody, the cells were incubated with 100 µl of 50% PBS/normal goat serum (NGS; Gibco) or rabbit non-immune serum to block non-specific retention of the first antibody. After removal of the blocking agent, the cells were further incubated with the first antibody (100 µl or 50 µl) diluted in 50% PBS/NGS + 0.02% sodium azide.

The first antibody dilutions were as follows:

a)	anti-fibronectin	Dr. R. Morris	1:100
b)	anti-GFAP	Prof. M. Raff & Dr. A. Bignami	1:100 - 1:200
c)	anti-galactocerebroside	Dr. B. Ranscht	1:50

d)	anti-Thy-1 (OX-7)	Dr. A. Williams 1:100
e)	anti-neurofilament protein (RT97 clone supernatant)	Dr. B. Anderton Undiluted - 1:150
f)	anti-glutamine synthetase	Dr. G. Pilkington 1:10 - 1:50
g)	anti- γ -glutamyl transpeptidase	Dr. S. Murphy 1:50
h)	Chagasic serum	Dr. G. Wilkin 1:20

At the end of the incubation, the antibodies were removed and cold washed with PBS as before. In order to block non-specific retention of the fluorochrome-linked second antibody, the cells were again incubated with 100 µl of 50% PBS/NGS for 10 minutes before the addition of the second antibody (50 µl for 20 minutes). For polyclonal antisera raised in rabbits the second antibody was goat anti-rabbit rhodamine conjugate (Miles Immuno-chemicals Ltd.); for monoclonal antibodies, goat anti-mouse rhodamine (Nordic) and for Chagasic serum, goat anti-human fluorescein (a gift from Dr. G. Wilkin) was used. All second antibodies were used at 1:40 or 1:50 diluted in 50% PBS/NGS + 0.02% sodium azide, except goat anti-human fluorescein which was used at 1:20.

After incubation with the second antibody, the cells were again washed with 2 - 3 ml PBS. Where extracellular antigens were being labelled the cultures were now fixed as previously noted. The coverslips were then mounted onto glass slides in glycerol sealed onto the slide using clear nail varnish.

For double-labelling experiments, the above procedure was followed first for labelling of extracellular antigens then, following fixation, repeated for intracellular antigens.

The controls used for immunofluorescence studies were the omission of the first antibody, its replacement with non-immune rabbit serum or the complete absence of any antibodies or sera except for 50% PBS/NGS.

The labelled cells were then viewed using a Zeiss Standard microscope fitted with a VI FL fluorescence unit containing selective filter sets for rhodamine and fluorescein fluorescence. All photographs of these cells were taken using a Nikon camera attachment and 400 ASA Kodacolour or 125 ASA Ilford FP4 film.

Immunodotting

Immunodotting experiments were performed on homogenates prepared from primary culture of astrocytes as in Chapter 2. Briefly, in two separate experiments, a culture grown in a 60 mm diameter dish was washed twice with PBS and the cells harvested into 1 ml of distilled water at 4°C and sonicated on ice (10 sec. at an amplitude of 12, Soniprep, MSE Scientific Instruments). An aliquot was taken for determination of protein content (Lowry et al, 1951) and then approximately 2 µg and 0.2 µg protein were dotted onto nitrocellulose paper grids in duplicate. The procedure for visualisation of antigens using the antibodies stated below was as described in chapter 2.

a) anti-GFAP (Prof. M. Raff) 1:300

b) anti-fibronectin (Dr. R. Morris) 1:200

c) anti-galactocerebroside (Dr. B. Ranscht) 1:400

d) anti-Thy 1 (Dr. R. Morris) 1:125

e) anti-neurofilament protein (Dr. B. Anderton) 1:10

f) anti- γ-glutamyl transpeptidase (Dr. S. Murphy) 1:50

Glutamine synthetase assay

Confluent astrocyte cultures grown in 60 mm diameter petri dishes were assayed for glutamine synthetase essentially as in Ward and Bradford (1979). The cells were washed twice with 3 mls of ice cold 0.9% NaCl and harvested into 2 ml of buffer containing imidazole buffer (150 mM, pH 7.2), $MgCl_2$ (19 mM) and mercaptoethanol (30 mM) and sonicated on ice (10 sec. at an amplitude of 12, Soniprep, MSE Scientific Instruments). Then 100 µl aliquots of disodium ATP (30 mM), monosodium glutamate (1.5 M), hydroxylamine (3 M freshly neutralised to pH 7.2 with KOH) and pyruvate kinase (500 units ml^{-1}) were added, together with 300 µl aliquots of ouabain (10 mM) and phosphoenol pyruvate (130 mM). The mixture was incubated at 37°C for 30 minutes at the end of which, duplicate 400 µl aliquots were taken and added to 800 µl of a mixture of $FeCl_3$ (50 mM), perchloric acid (400 mM) and HCl (400 mM). After standing on ice for 30 minutes the protein precipitate was pelleted by centrifugation at 12,400 g for 10 minutes (Microfuge 12, Beckman Instruments) and the optical density of the supernatant was read at 500 µm. Standard curves were constructed by the addition of 10 - 1000 nmoles of γ-glutamylhyroxamate to the $FeCl_3$ mixture.

Further enrichment of astrocyte cultures

Three possible methods for the further enrichment of the cell cultures with astrocytes were evaluated:-

a) Cytosine Arabinoside

The first of these was essentially that of Rougon et al (1983). Astrocyte cultures were grown on poly-D-lysine coated coverslips as

previously described. At the first medium change (6 d.i.v.), 6 cultures were treated with 20 μM cytosine arabinoside and 6 cultures left untreated as controls. After 48 hours the cultures were washed twice with 0.5 ml of fresh medium and left to grow to confluence. No further medium changes were made.

b) Thy 1 immunocytolysis in suspension

The destruction of Thy 1^+ cells in suspension by immunocytolysis prior to cell culture was attempted. Three aliquots of cells (3.5 - $5x10^6$) were taken from the cell suspension produced during a normal culture preparation. These aliquots were incubated in medium ± 25 μg of anti-Thy 1 (OX-7) for 30 minutes at room temperature and then washed twice in fresh medium by centrifugation at 600 r.p.m. for 10 minutes. After washing, the cells were resuspended in 200 μl of medium ± 100 μl guinea pig complement or medium alone and incubated for 45 minutes at 37°C in a shaking water bath. Again, the cells were washed twice in fresh medium after which the cells were counted and plated onto 13 mm coverslips as before.

c) Immunocytolysis in situ

Finally, the feasibility of using immunocytolysis to kill cells in confluent cultures in situ was examined by the use of B1 and anti-GGT antiserum. In short, astrocyte cultures grown on coverslips were treated with 25μg of B1 or anti GGT in 0.5 ml of medium for 20 minutes at room temperature. The medium was then removed and the cultures washed with 0.5 ml of fresh medium which was replaced with 300 μl of medium with or without 2:1 guinea pig complement. The cultures were then incubated for 45 minutes at 37°C. After removal of the guinea pig complement, the cultures were washed once with 0.5 ml of fresh medium and left for one

week in 0.75 ml of medium when they were examined under the microscope.

Results

Astrocyte-enriched cultures

The method for producing astrocyte-enriched cultures from the neontal rat cortex produces an initial yield of approximately 15 million cells per animal at all ages used (0 - 2 days) Table 3.1. Cellular debris mounted to some 30% of the total particle count. The cells begin to put out processes after 2 - 3 days in vitro and divide and enlarge to produce confluent cultures by 18 - 22 d.i.v., with a protein content of between 300 - 800 µg per 60 mm dish (mean 480 ± 13 µg, n = 205). A representative field of a confluent astrocyte culture at 19 d.i.v. is shown in Fig 3.1. The most common cell type is of a flattened polygonal morphology. Observations of the cultures under the light microscope suggests that there are no neurons, defined as ovoid cells with one or two fine processes. Similarly, there are no cells with beating cilia which are commonly regarded as ependymal cells. However, two cells types other than flattened polygonal cells are seen in these cell culture, e.g. densely packed columnar cells which form loops or whorls. These cells are very similar to those seen in Fig. 3.2, a micrograph of a confluent meningeal cell culture. Also, rounded phase bright cells with a granular cytoplasm are seen. These cells readily take up Indian ink and are probably macrophages.

Immunodotting shows that the most prominent antigens in

Table 3.1 Yield of cells from neonatal cortices

Age (days)	Cells ($x10^6$) ± s.e.m.		% Debris ± s.e.m.	
0	15.2 ± 0.6	(23)	31 ± 1	(23)
1	14.9 ± 1.2	(15)	28 ± 2	(15)
2	14.7 ± 1.3	(10)	34 ± 1	(10)

Legend

The yield of cells in suspensions of neonatal rat cortices was estimated as described in Methods using a Coulter Counter. The number of cells was determined by subtracting the amount of debris (particles under 6 μm) from the total number of particles present (particles above 6 μm). The numbers in brackets refer to the number of individual culture preparations; all counts were made in triplicate.

Fig. 3.1

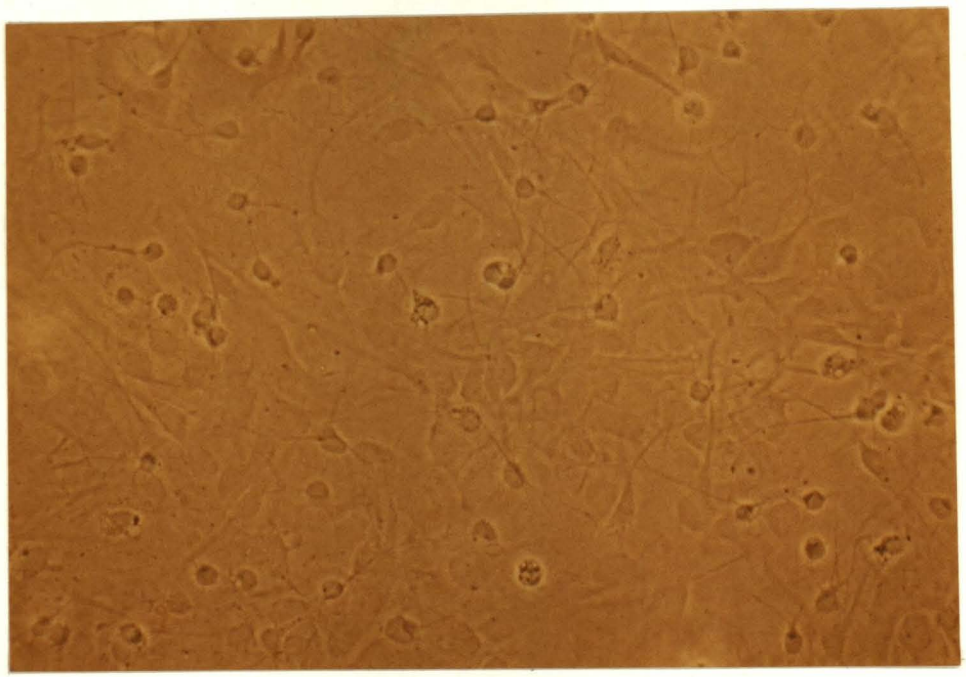
Astrocyte-enriched culture

A phase contrast micrograph of a representative astrocyte-enriched culture at 19 d.i.v., x 640. A large processed astrocyte (Lp) is indicated together with a macrophage (M) against a background of predominantly flattened, polygonal astrocytes.

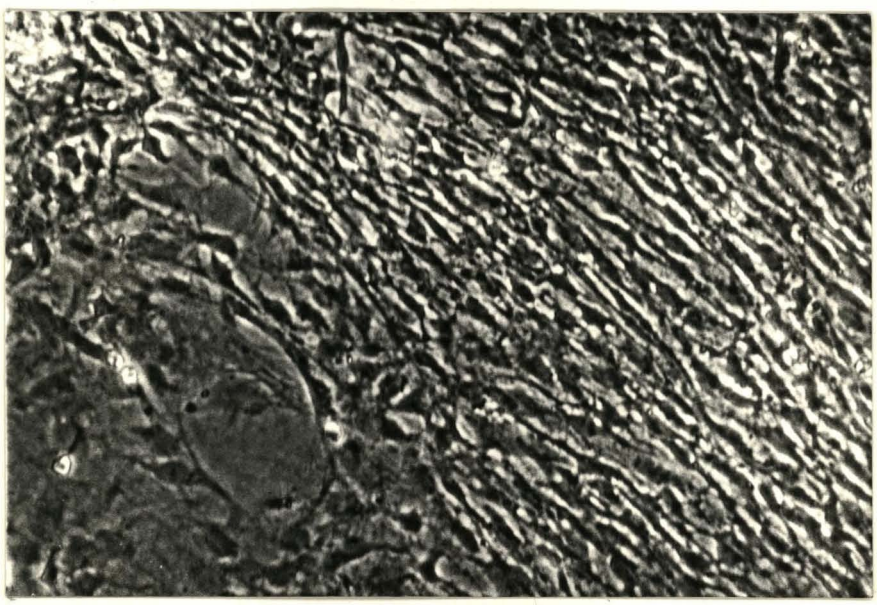
Fig. 3.2

Meningeal cell culture

A phase contrast micrograph of a representative meningeal cell culture at 14 d.i.v., x 500. Densely packed columnar cells (col) are observed together with flattened polygonal cells (Fp).



M Lp



Col

Fp

homogenates prepared from confluent astrocyte cultures are GFAP and FN (Figs 3.3 and 3.4). Neurofilament protein, galactocerebroside (GC) and Thy 1 immunoreactivity are almost completely absent; the only other antigen which is positively identified in the cultures is γ-glutamyl transpeptidase (GGT).

The use of the immunofluorescent technique to identify antigens expressed by individual cells in astrocyte cultures shows that the cultures stain extensively for GFAP (Fig. 3.5) and that the cells labelled with GFAP are of at least three different morphological types. Most common are large flattened polygonal cells (Fig 3.5). However, large processed cells (Fig. 3.6) and smaller processed cells (Fig. 3.7) are also seen. Examination of GFAP immunofluorescence staining of astrocyte cultures might suggest that the cells were almost exclusively 100% $GFAP^+$. However, FN is also present in these cultures, and is found in both cell-associated and non cell-associated forms. Fig. 3.8 illustrates the most common staining pattern for FN and shows a densely packed, multilayered grouping of cells. These cells conform to the columnar cell type seen in Fig. 3.2. Due to the dense packing of the FN^+ cells, no count of the number of cells was possible. Cells of this morphology are also seen to label with anti-Thy 1, although the intesity of labelling was variable. FN is also present in a non cell associated form (Fig. 3.9) where it forms 'trails' or very rarely 'mats' (Fig. 3.10). Additionally a weak but specific intracellular perinuclear staining of FN is observed in most cells which do not express the very marked cell associated staining pattern.

Fig. 3.3

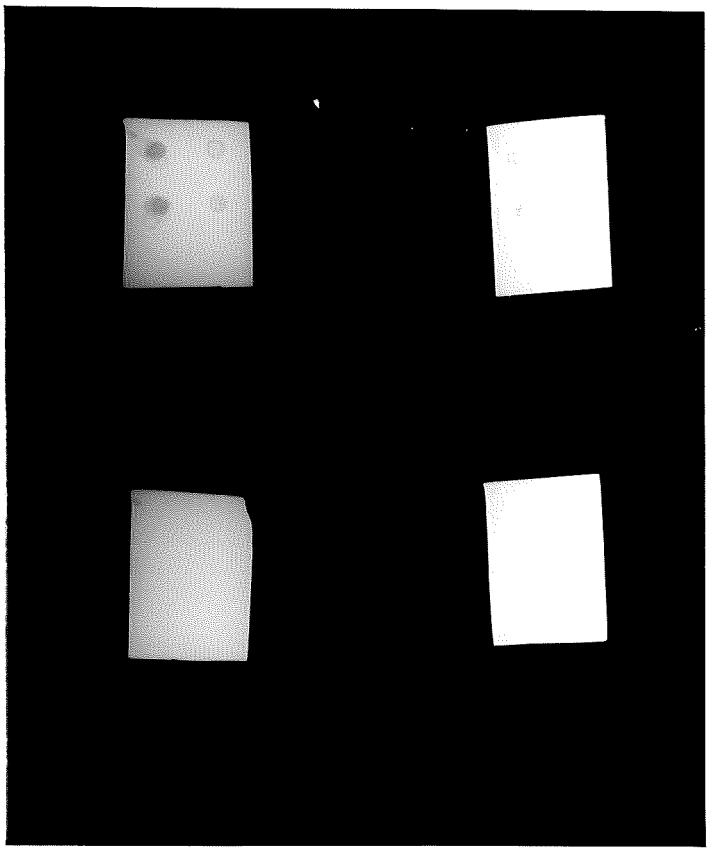
Characterisation of astrocyte-enriched cultures by immunodotting I

Immunodots of duplicate 2.0 and 0.2 µg aliquots of astrocyte-enriched culture homogenates using anti-GFAP, anti-FN, anti-GGT and pre-immune rabbit serum.

Fig. 3.4

Characterisation of astrocyte-enriched cultures by immunodotting II

Immunodots of duplicate 2.0 and 0.2 µg aliquots of astrocyte-enriched culture homogenates using anti-Thy-1, anti-GC, anti-neurofilament protein (NF) and no 1st antibody.


GFA
FN
C
GGT

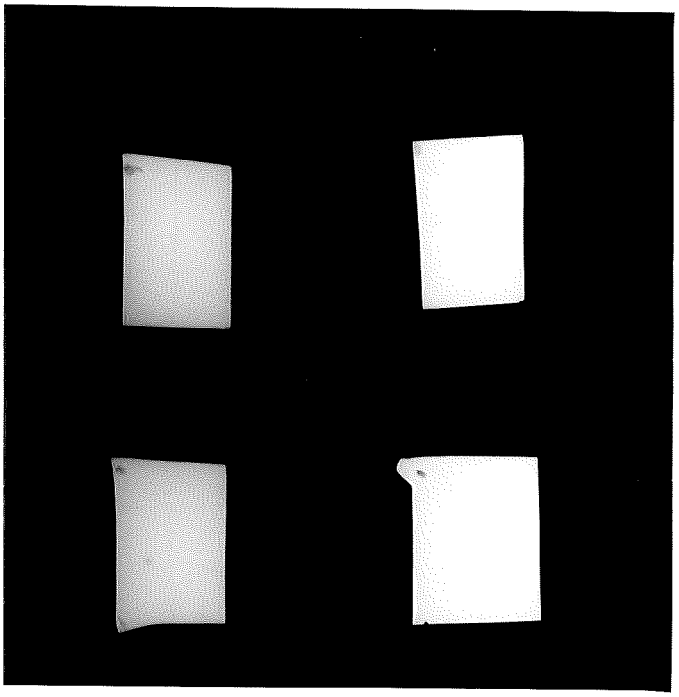

GC
NF
C
THY 1

Fig. 3.5

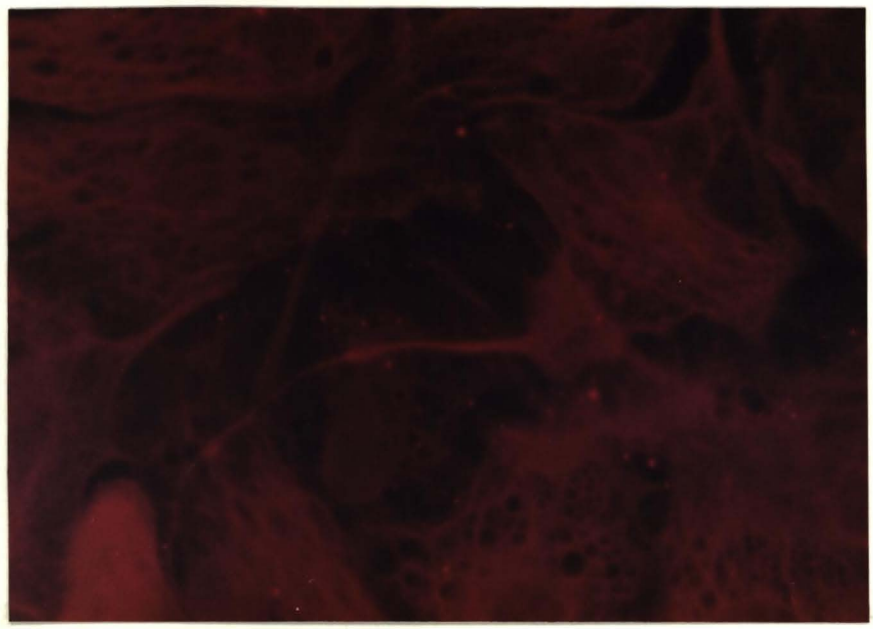
GFAP immunofluorescence micrograph I

Flattened polygonal cells in astrocyte-enriched cultures labelled with antiserum to GFAP, x1200.

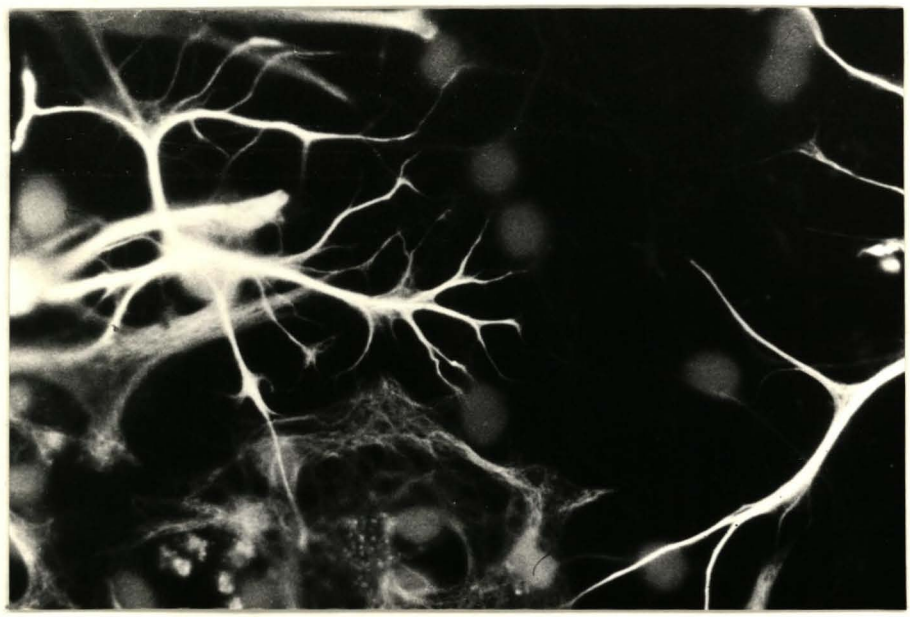
Fig. 3.6

GFAP immunofluorescence micrograph II

Highly fluorescent, large, multiprocessed, $GFAP^+$ cells (Lp) and flattened polygonal $GFAP^+$ cells (Fp) in an astrocyte-enriched culture, x1200



Lp



Fp

Fig. 3.7

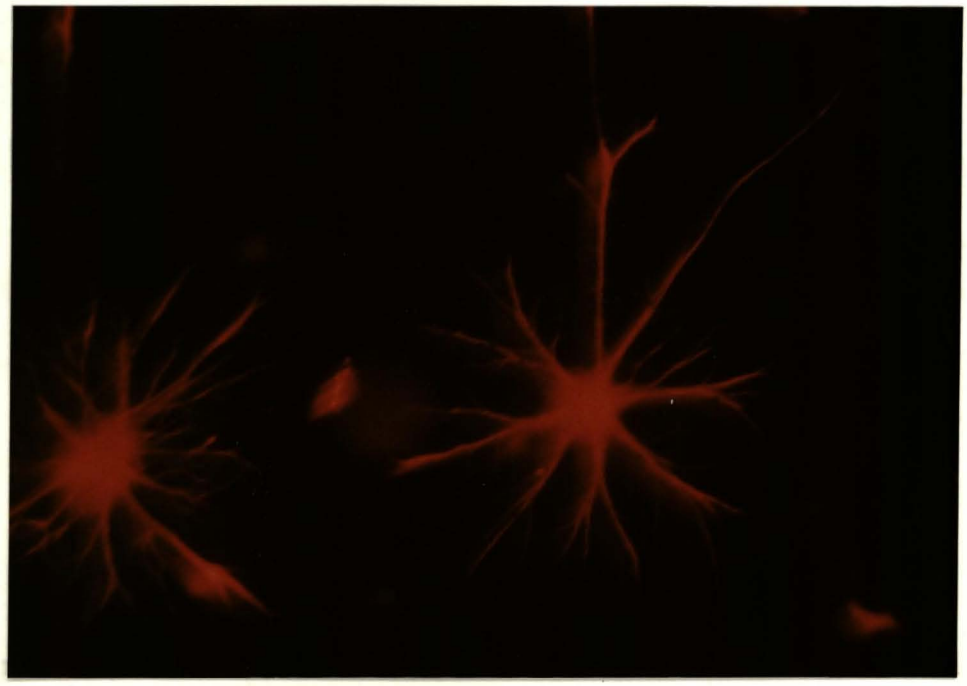
GFAP immunofluorescence micrograph III

Finely processed $GFAP^+$ cells in astrocyte-enriched cultures, x 2000.

Fig. 3.8

Fibronectin immunofluorescence micrograph I

Dense, multilayered aggregation of FN^+ cells underlying FN^- cells in an astrocyte-enriched culture, x 1400.



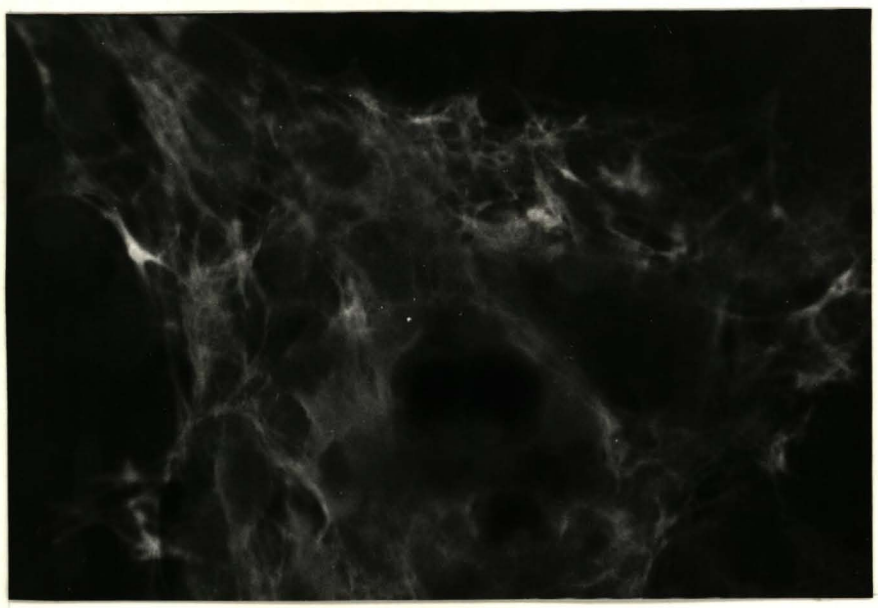


Fig. 3.9

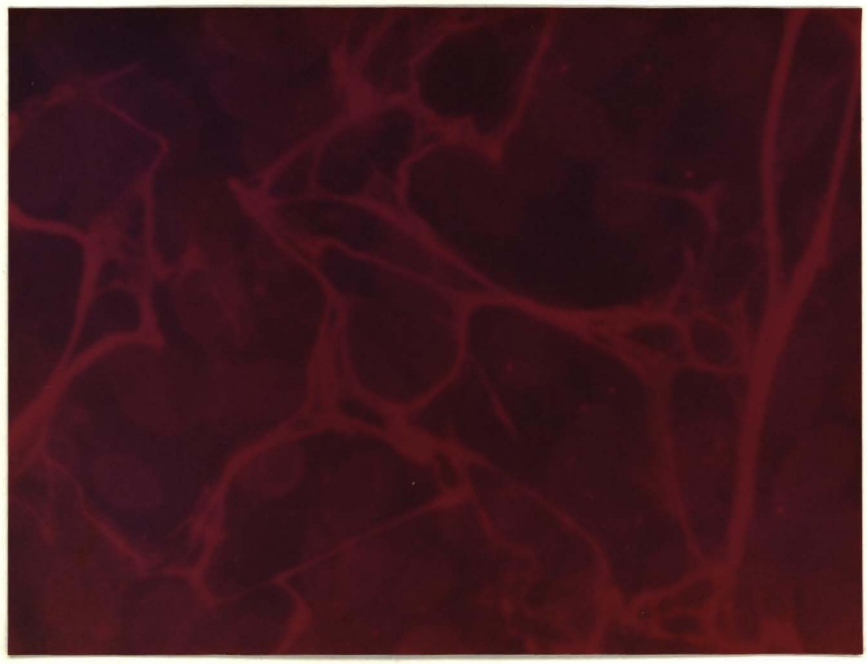
Fibronectin immunofluorescence micrograph II

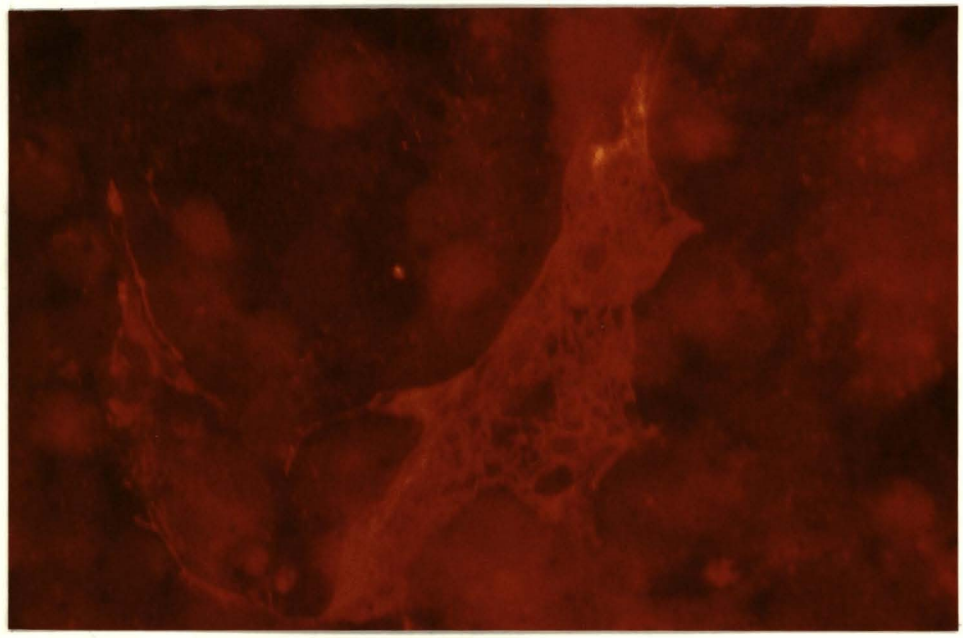
Non cell-associated FN "trails" in astrocyte-enriched cultures underlying cells with faint cytoplasmic, perinuclear labelling FN antiserum, x 1400.

Fig. 3.10

Fibronectin immunofluorescence micrograph III

Non cell-associated FN "mat" observed in an astrocyte-enriched culture, x 1400.





It is important to note that whilst whorls of columnar cells, which are observed to be the major cell type associated with FN in astrocyte cultures, can be found in such cultures, routine examinations of the cultures under the light microscope showed that whorls of cells are by no means always present in a particular culture dish. Further, the degree of contamination varies between different culture preparations. Although the densely packed and multilayered nature of the FN^+ columnar cells prohibits an accurate cell count, examination of more than 10 separate cultures using FN immunofluorescence and routine light microscopic observation of astrocyte cultures suggests that whorls of columnar cells commonly account for no more than 10 - 15% of the total number of cells present. Moreover, when cultures contained more than this approximate amount they were discarded.

Thy 1^+ cells (other than columnar cells) were present in these cultures and represented 10% of the total number of $GFAP^+$ cells in one experiment. In a separate experiment however, this figure appeared to approach 50% or more Thy 1^+ cells. Thy 1^+ cells other than those of the morphology labelled with FN were of a flattened polygonal appearance.

GC^+ cells were very rarely seen in astrocyte cultures. Similarly no cells which were neurofilament $protein^+$ were observed. An antiserum to GGT, a supposed capillary cell marker, labelled the surface of all cells in astrocyte enriched cultures as did the antiserum B1 as previously mentioned (Chapter 2.)

Serum from humans with Chagas' disease when added to unfixed cultures produced a subsequent pattern of widely scattered dots of

fluorescence but control cultures labelled with anti-human fluorescein alone also produced a similar pattern though not as dense. The labelling of cells with Chagasic serum and other surface labelling antibodies proved difficult to reproduce photographically, partly due to the high level of background autofluorescence seen in these dense cell cultures. Control slides either incubated with no first antibody or with pre-immune rabbit serum also showed a high level of autofluorescence which could not be reduced using alternative fixatives e.g. methanol or 4% paraformaldehyde, or a different mounting agent e.g. DPX (BDH Biochemicals). Non-specific fluorescence i.e. that fluorescence seen in control slides which was not autofluorescence, was very low in most cases.

Cultures incubated in the absence of any cell specific antibodies sometimes contained cells which were labelled with all the fluorescence conjugated second antibodies used. These cells had the appearance of the phase bright, rounded, granular cells previously seen to take up Indian ink. They were considered to be macrophages and were present in cultures to a varying degree, from almost total absence to approximately 11% of total cells. However, the macrophages were loosely attached to the surface of the cultures and washing the dishes with 2 x 3 mls of PBS with swirling was sufficient to lower this value to approximately 2%. Generally macrophage contamination was low and the numbers remaining after washing prior to use in the experiments described later were negligible.

An antiserum to glutamine synthetase (Dr. G. Pilkington) showed no specific staining. In contrast, when homogenates of

astrocyte enriched cultures were assayed for glutamine synthetase an activity of 0.95 ± 0.11 μmol/mg/hr (n = 14) was measured.

Enrichment of astrocytes in cell cultures

Three methods were assessed for their possible routine use in purifying astrocyte cultures further. None were found to be useful. Treatment of the cells with cytosine arabinoside resulted in the almost total destruction of all cells present. Similarly, no cells which were treated during separation of the neonatal cell suspension, prior to plating, with anti-Thy 1 and complement, complement alone or those cells just subjected to the immunocytolysis procedure, survived in culture. Further, microscopic examination of confluent astrocyte cultures treated with B1 or anti-GGT in combination with complement showed no lysis of cells and no difference between antibody and complement treated, complement treated or untreated cultures.

As a result of these three preliminary experiments and the results from the characterisation of the cell cultures, a method was devised for the growth of meningeal cells in vitro to use as a control for all future experiments using astrocyte-enriched cultures.

Meningeal cell cultures

Fig. 3.2 shows a phase contrast micrograph of a confluent culture of meningeal cells at 14 d.i.v. After initial plating, the meningeal cells grow and divide rapidly producing confluent

at 11-15 d.i.v. containing 300-1200µg protein, mean 512±23µg (n=216). Confluent meningeal cell cultures if left until 18-22 d.i.v., the age at which astrocyte enriched cultures are used, have a tendency to detach from the culture dish.

The cell types seen in meningeal cultures show some similarities to those in astrocyte-enriched cultures i.e. cells of flattened, polygonal shape and cells of columnar morphology organised into closely packed "whorls". The columnar cells are generally more numerous in meningeal cell cultures than in astrocyte cultures although the abundance of these cells varies widely. No ciliated cells and very few macrophages are present in these cultures.

Immunodotting experiments performed in our laboratory using antibodies to FN, GFAP, GGT and THY - 1 (Murphy, unpublished observations) have shown that the most prominent antigens present in homogenates of meningeal cells in vitro are FN and GGT. There is some Thy 1 immunoreactivity and notably some GFAP is also present which indicates that there are some astrocytes present,although the labelling is markedly less than in astrocyte cultures

Discussion

A comparatively simple method for the production of astrocyte enriched cultures from the cortex of the neontal rat has been described together with the results of extensive characterisation of these cultures. The method has many advantages over the adult cell separation technique described in Chapter 2. The brain of one newborn rat yields five confluent cultures after 18-22 d.i.v. which represents on average 2400 µg of protein in total. This is compared to 200 µg of protein which was the yield of the astrocyte fraction from one adult rat cortex using the cell separation method. Sufficient material for several biochemical experiments on viable, intact cells can be obtained from one culture preparation compared to the very limited scope for experimentation on adult cells prepared by a very long separation process. Furthermore the cultures are astrocyte enriched (approximately 85% astrocytes) which could not be stated positively for the material in the fraction derived by the adult cell separation method. Finally, the contaminating cells present in the astrocyte enriched cultures could be identified and attempts made to control for them.

Immunodotting studies showed that GFAP, an astrocyte specific protein in vivo thought to be associated with glial filaments (Bignami and Dahl, 1974; Schachner et al, 1977) was the most prominent antigen in astrocyte culture homogenates. Similarly, immunofluorescence experiments show an intense labelling of the cells with anti-GFAP antiserum which, if taken in isolation, would suggest that $GFAP^+$ cells comprised almost all of the cells present. Indeed the culture conditions were chosen to favour the growth of glia in general and astrocytes in particular. In the rat

cerebral cortex at birth, neurons have, in the main, ceased dividing and glial cells are the most mitotically active cell type (see e.g. Parnavelas et al, 1983). Moreover the concentration of K^+ in the culture medium (<5mM) is known to be unfavourable for neuronal growth in vitro (Lasher and Zagon, 1972). The seeding density of the initial cell suspension was low to reduce the proliferation of oligodendrocytes (see Kimelberg, 1983). Furthermore, the presence of serum in the culture medium is known to favour the differentiation of astrocytes rather than oligodendrocytes from a common precursor cell in optic nerve cultures (Raff et al, 1983). In addition, chick embryo extract was used in an attempt to inhibit the growth of fibroblasts as described by Coogan et al (1968).

The expression of GFAP by the cells in culture not only shows that they are astrocytic but also, as astrocytes in vivo mostly express GFAP at myelination (at the second or third post-natal week; Dahl, 1981), that at least in one respect the astrocytes are differentiated.

The specific activity of glutamine synthetase (0.95 ± 0.11 µmol/mg/hr) an astrocyte specific enzyme in vivo (Norenberg and Martinez-Hernandez, 1979) is similar to other values previously reported for astrocyte cultures after three weeks in vitro (Hallermayer et al, 1981; Tardy et al, 1982; Juurlink, 1982). However, it must be noted that this level of activity is not as high as is seen in homogenates prepared from adult rat cortex e.g. 2.5 µmol/mg/hr (Patel et al, 1983). These results indicate that, while the cell cultures obtained using this method contain predominantly astrocytes and may be differentiated in some aspects of their biochemistry, they do not appear to be identical to astrocytes in vivo.

The morphology of the GFAP$^+$ cells was predominantly of a flattened polygonal appearance (Fig. 3.5), resembling the Type 1 astrocytes described by Raff and co-workers (see Temple and Raff, 1985). Large multiprocessed GFAP$^+$ cells (Fig. 3.6) were also usually present in the cultures although their number varied quite widely and no cell counts were attempted. Smaller, processed GFAP$^+$ cells which resembled, morphologically, Type II astrocytes as described by Raff and co-workers (see Temple and Raff, 1985) were comparatively rare in the astrocyte cultures used here. These cells were usually found at the edges of the coverslips on which the cells were grown (Fig. 3.7) and were not in contact with other cells. However, on rare occasions it was possible to observe small, processed GFAP$^+$ cells in close conjunction with larger flattened GFAP$^+$ cells in the confluent areas of the cell cultures even after three weeks in vitro. Such small GFAP$^+$ cells had clearly not changed into large, polygonal astrocytes on contact with other GFAP$^+$ cells as is believed to occur in cerebellar astrocyte cultures (Wilkin, pers. comm.)

When astrocyte cultures were labelled with anti-FN, a proposed cell specific marker for meningeal cells, endothelial cells and fibroblasts in vitro (Schachner et al, 1978; Raff et al, 1979), it became clear that $GFAP^+$ cells, whilst in the majority, were not the only cell-type present. Immunodotting studies showed that FN was a prominent antigen in homogenates of astrocyte cultures. Also, cells which expressed FN on their surface were observed in astrocyte cultures using immunofluorescence. These FN^+ cells were arranged in densely packed bundles or whorls, were of a columnar shape and were readily identified under the light microscope. Cells of this morphology also label with anti-Thy 1, although with varying intensity. This is not in agreement with the results from anti-Thy 1 immunodotting (Fig. 3.4) but probably reflects the use of different antibodies. FN^+, Thy 1^+ cells in CNS cultures are thought to be meningeal fibroblasts (Raff et al, 1979) whereas FN^+ Thy 1^- cells were taken to be pial and arachnoid cells from the meninges, although this distinction is unclear (Bartlett et al, 1981).

The number of FN^+Thy 1^+ meningeal fibroblasts in astrocyte cultures was very difficult to assess due to their dense local packing Considering (a) the variation in the number of columnar cells in astrocyte cultures as seen during routine examination, (b) the degree of cell associated FN staining and (c) the fact that cultures with high proportions of columnar cells were discarded, an estimate of 10-15% FN^+ cells in astrocyte cultures is given. Moreover, the biochemical experiments described in Chapters 4, 5 and 6, in particular the differential effects of $[K^+]_o$ and neurotransmitter agonists on glycogen stores of astrocytes and meningeal cells, strongly suggest a minimal meningeal cell contamination in astrocyte cultures.

FN staining not associated with a particular cell type is also observed in astrocyte cultures. This may be derived from the serum present in the culture medium as the antiserum used was raised against blood serum fibronectin which cross reacts with extracellular matrix fibronectin (Dr. R. Morris, pers. comm.; Yamada and Olden, 1978). Alternatively, the trails of FN may be left by migrating FN^+ cells.

Anti-FN was also observed to label intracellularly the majority of cells present in astrocyte-enriched cultures. This result was unexpected as astrocytes are not reported to express FN (Raff et al, 1979). As astrocytes are known to take up serum proteins in vivo (Trachtenberg, 1983), and as the anti-FN antiserum used here was raised against serum fibronectin, the intracellular staining in astrocyte cultures may represent labelling of endocytosed serum fibronectin.

Anti-Thy 1 also labelled cells of a flattened, polygonal morphology, (a morphology not seen to be labelled with anti-FN), in addition to the FN^+ columnar cells. These cells were taken to be Thy 1^+, $GFAP^+$ astrocytes which are know to occur in astrocyte cultures after 1 week in vitro (Raff et al, 1979) and may consitute 58% of the total cells present (Pruss, 1979). Double-labelling experiments using anti-GFAP and anti-Thy 1 proved unsuccessful due to the detachment of the majority of cells during the procedure.

Visual examination of the cultures indicated the absence of neurons. In agreement with this is the complete absence of any positive reaction with anti-neurofilament protein antiserum in

immunofluorescence or immunodotting studies. This result was expected as rat astrocyte cultures prepared using a variety of similar methods are never reported to contain neurons and the culture conditions had been chosen to inhibit the survival of neurons. Notably, Hansson et al (1980) state that neurons are absent from rat hemisphere cultures on the basis of cell morphology and, moreover, the absence of cells positive for 14-3-2 protein, i.e. neuron specific enolase (Marangos et al, 1976; Langley et al, 1980), in immunofluorescence studies.

As expected from the choice of culture conditions GC^+ cells (oligodendrocytes; Raff et al, 1978) were very rarely observed in astrocyte cultures, in spite of a slight immunoreactivity seen in immunodotting experiments. This is probably due to the removal of these cells by the washing process involved in the immunofluorescence procedure as they are attached to the surface of mixed glial cultures and may be removed by shaking the cultures (McCarthy and de Vellis, 1980).

Macrophages, identified as round, phase bright granular cells which accumulated Indian ink and fluoresced after exposure to any fluorochrome linked antibody, were also readily removed from the cultures by washing.

Capillary endothelial cells which have been reported to contaminate astrocyte cultures (Hansson et al, 1980; Woodhams et al, 1981) could not be positively identified by Chagasic serum in the culture preparation used here. Chagasic serum is derived from patients with Chagas' disease, a South American trypanosomiasis caused by T. cruzi, and has been reported to label capillary

endothethial cells in vivo and in vitro (cf Wilkin et al, 1981). In addition, no FN^+ cells could be identified with either of the morphologies ascribed to capillary endoethelial cells, i.e. small (10 µm perikaryon) cells with flattened velate processes (Wilkin et al, 1981) or more flattened, polygonal cells with short processes (Hansson et al, 1980). It appears however that there may be some capillary endothelial cells present in these astrocyte cultures as there is low but measurable alkaline phosphatase activity in astrocyte cultures (0.18 ± 0.01 µmol/mg/hr, Murphy unpublished experiments). Alkaline phosphatase is believed to be localised to capillary endothelial cells (Kange et al, 1978) although there is evidence for its presence in neurons (Sugimura and Mizutani, 1979). The activity of the enzyme in rat cerebral microvessels is however markedly higher (7.4 µmol/mg/hr, Estrada et al, 1983) than in astrocyte cultures. GGT as visualised by immunofluorescence is found to be present on all cells in the astrocyte cultures and its use as a capillary endothelial cell-specific marker in vitro (DeBault and Cancilla, 1980) must be questioned.

Extensive characterisation of astrocyte-enriched cultures (as used for larger biochemical experiments) thus showed that $GFAP^+$ cells predominate and that the major contaminating cell type(s) are FN^+ and probably derived from the meninges. A series of preliminary experiments designed to increase the astrocyte content of the cultures were unsuccessful.

Cytosine arabinoside treatment, as described before, destroyed all cells present in the cultures. It is possible that replating the treated cells as performed by Rougon et al (1983) may be crucial for the success of this technique. This would have been an unrealistic procedure to add to the method described for producing

large numbers of astrocyte cultures.

The use of anti-Thy 1 and complement to destroy Thy 1^+ cells (fibroblasts) in adult astrocyte culutres has been described by Lindsay et al (1982) but there are limitations on the use of this method to remove Thy 1^+ cells in astrocyte cultures derived from the neonatal cortex. For example, the levels of Thy 1 in rat brain are very low at birth (Schachner and Hammerling, 1974) and its exact cellular distribution at this stage is unknown. Thus it is possible that the FN^+, Thy 1^+ cells seen in confluent cultures of astrocytes may not be those cells which express Thy 1 at birth. However, this technique was used in an attempt to destroy Thy 1^+ cells in a suspension of cells from the neonatal rat prior to cell culture. The procedure followed was apparently too rigorous for the cells at this stage, (just after disaggregation of the cortex), as no cells, control or experimental, survived in culture.

As a result of the theoretical and methodological problems involved in immunocytolysis of Thy 1^+ cells in neonatal cortex cell suspensions and the infeasability of replating large numbers of cultures, the possibility of destroying Thy 1^+ or FN^+ cells in confluent astrocyte cultures in situ was examined. It must be noted, however, that some of the Thy 1^+ cells at this stage are probably also $GFAP^+$, so successful treatment with anti-Thy 1 and complement would destroy some astrocytes as well as fibroblasts. For the initial study, B1 and anti-GGT were used as both of these were more available than either anti-Thy 1 or anti-FN and both were known to label most cells in astrocyte cultures. Thus if the concept of immunocytolysis in situ itself was valid, then incubating confluent astrocyte cultures with these antisera and complement should produce cell lysis. In

fact no cell death was observed in either antibody and complement treated cultures. This suggested that in order to remove Thy 1^+ or FN^+ cells, cultured cells might have to be removed from the culture dishes into a suspension, lysed and then replated. This, as previously noted, was not practicable for large numbers of cultures.

Astrocyte-enriched cultures prepared by this method therefore contain a population of FN^+ cells, presumably derived from the meninges, and these are not readily removed. As these culture were to be used as a model for astrocytes in future biochemical studies it was important to allow for the contamination with other cell types. To this end a simple method for the culture of meningeal cells, the most probable contaminants, was developed. The method uses meninges removed from brains prior to use in preparing astrocyte enriched cultures and should represent closely those FN^+ cells which are known to contaminate astrocyte cultures. Indeed immunodotting experiments show that meningeal cell cultures contain substantial amounts of FN (Murphy, unpublished experiments). Furthermore, meningeal cultures may contain cell types which are also present in astrocyte cultures but not readily detected by immunocytochemical means. For example, astrocyte enriched cultures are commonly regarded as being contaminated with capillary endothelial cells as previously discussed. Immunofluorescence studies however did not provide conclusive evidence for the presence of these cells in astrocyte cultures. Conversely, the cultures contain alkaline phosphatase, an enzyme found predominantly in capillary endothelial cells. Meningeal cell cultures would appear to be a useful control for this and possibly other contaminating cell types as they too contain alkaline phosphatase activity (and thus presumably capillary

endothelial cells) and in greater amounts than in astrocyte cultures (0.30 ± 0.04 µmol/mg/hr against 0.18 ± 0.01 µmol/mg/hr). However, it must be noted that the level of alkaline phosphatase seen in meningeal cultures is rather less than that seen in isolated microvessels (7.4 µmol/mg/hr, Estrada et al, 1983) which may indicate that capillary endothelial cells do not survive well under these culture conditions.

In summary, a method for the production of astrocyte enriched cultures from the neonatal rat cortex has been described and these cultures have been well defined with regard to cell type. The method produces a culture system highly enriched in astrocytes as determined by GFAP immunoreactivity and the level of glutamine synthetase activity. The cultures also contain a variable population of FN^+ cells (estimated at 10 - 15%) which are thought to be FN^+, Thy 1^+ fibroblasts derived from the meninges. In order to control for the presence of contaminating cells in cultures that were to be used in experiments to determine the action of neuroactive agents on astrocytes, a method for meningeal cell culture was developed.

CHAPTER 4

The effect of changes in extracellular K^+ on astrocyte glycogen stores

Introduction

In Chapters 2 and 3, bulk separation of astrocytes from the adult rat cortex and rat cortical astrocyte cultures were evaluated as possible models to be used to examine the effects of putative neuron/astrocyte signals (Chapter 1). Due to the methodological problems involved in bulk cell separation, astrocytes in primary culture were selected. This chapter describes the use of astrocyte enriched cultures and cultures of meningeal cells, the cell type(s) considered as the most likely contaminant, to examine the effects of changes in $[K^+]_o$ on astrocyte glycogen stores.

Glycogen stores were chosen as a possible indicator of neuron/astrocyte signalling in the mammalian CNS for several reasons.

First, glycogen in the CNS is localised predominantly in astrocytes in both the adult (Sotelo and Palay, 1968; Phelps, 1971; Peters et al, 1976) and in the young or perinatal animal (Bruckner and Biesold, 1981). In fact, as stated previously, the possession of rich supplies of glycogen is a characteristic feature of astrocytes. However, it must be noted that in young rats glycogen is also found in the cells of the choroid plexus, meninges and occasionally in the growing processes of axons (Bruckner and Biesold, 1981). In addition, some neurons in the pons, medulla and spinal cord of the rat contain glycogen up to about post-natal day 24

(Borke and Nau, 1984). In the adult CNS, glycogen is observed in the meninges (Ibrahim, 1975) and in some neuronal processes (Sotelo and Palay, 1968; Koizumi, 1974) and perikarya (Mossakowski et al, 1968; Ibrahim, 1975; Takieuchi, 1975).

Secondly, although the amount of glycogen stored in the CNS is relatively small when compared to the liver, the turnover of brain glycogen is higher than that of the liver by several orders of magnitude (Ibrahim, 1975) and may therefore be important in the overall metabolism of the CNS. Alternatively, the high turnover rate of CNS glycogen may reflect a higher cell activity, and therefore energy demand, in the brain. The enzymes controlling the turnover of glycogen in the brain are the same as those in the liver (Fig. 4.1) and have been examined previously with respect to their presence, activity, localisation and to some extent their regulatory mechanisms (see Maker and Lehrer, 1972; Ibrahim, 1975 for reviews; also Passoneau and Crites, 1976).

Thirdly, it has recently been proposed that the glycogen stores of glial cells respond to the activity of neurons (Pentreath, 1982). Pentreath and co-workers have examined extensively the inter-relationships between neurons and glia in invertebrate preparations (Pentreath et al, 1985). Of particular interest have been the studies performed regarding the incorporation of [^{3}H]-2-deoxyglucose ([^{3}H]-2-DG) into the glycogen stores of the leech (Haemopis sanguisuga) abdominal ganglia and the buccal ganglia of the snail, Planorbis corneus (Kai-Kai and Pentreath, 1981; Pentreath and Kai-Kai, 1982; Pentreath et al, 1982). Antidromic stimulation of the nerves in the ganglia resulted in an increased incorporation of [^{3}H]-2-DG into glycogen of glial cells and to a lesser extent neurons.

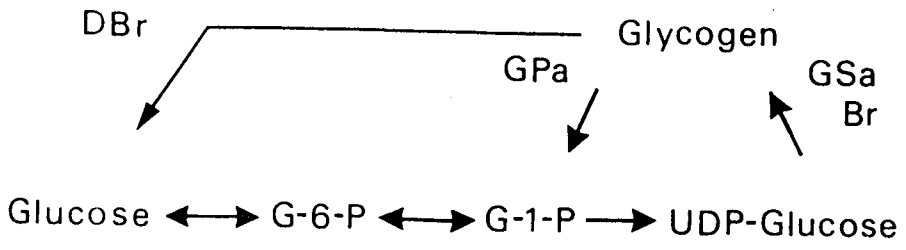
Fig. 4.1

Diagrammatic representation of glycogen metabolism in the liver

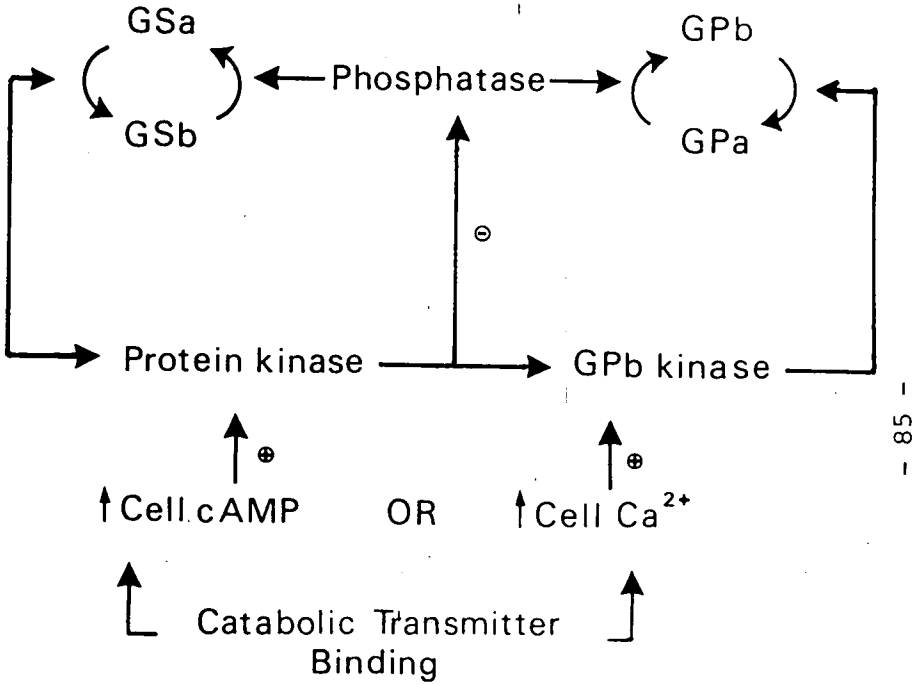
a) The synthesis of glycogen is catalysed by glycogen synthetase (GS; E.C.2.4.1.11) which transfers the glycosyl group of uridine diphospho-glucose (UDP-glucose) to the end of the glycogen chain. Glucose may also be added to the chain through the action of a branching enzyme (Br; E.C.2.4.1.18) which incorporates approximately 9% of the glycose found in glycogen. Glycogen is degraded by glycogen phosphorylase (GP; E.C.2.4.11) and a debranching enzyme (DBr; E.C.2.4.1.25) to yield glucose-1-phosphate and glucose respectively. Both of the principle synthetic and degradative enzymes are found in active (a) and inactive (b) forms.

b) The interconversion of the a and b forms of GS and GP is controlled by several enzymes whose activities are influenced by the levels of the intracellular second messengers cAMP and Ca^{2+}. Rising levels of cAMP activates protein kinase which evokes a reduction in glycogen synthesis by promoting the conversion of GSa to GSb and inhibiting the production of GSa from GSb by phosphatase. Also, increase in intracellular cAMP or Ca^{2+} promote the conversion of GPb to GPa through activation of GPb kinase and inhibition of phosphatase. Thus both the synthetic and degradative pathways of glycogen metabolism resulting in a net breakdown of glycogen stores.

A


DBr
Glycogen
GPa
GSa
Br
Glucose
G-6-P
G-1-P
UDP-Glucose

B


GSa
GSb
Phosphatase
GPb
GPa
⊖
Protein kinase
GPb kinase
⊕
⊕
Cell cAMP
OR
Cell Ca2+
Catabolic Transmitter
Binding

Similarly, the levels of glycogen formed from [^{3}H]-2-DG ([^{3}H]-2-deoxyglycogen) extracted from stimulated ganglia were increased 2 - 3 times over control ganglia (Pentreath and Kai-Kai, 1982). The incorporation of [^{3}H]-2-DG into ganglionic glycogen was also increased by elevated $[K^+]_o$, as seen by electronmicroscope autoradiography and measured by extraction of [^{3}H]-2-deoxyglycogen. Notably, the maximal increases in [^{3}H]-2-deoxyglycogen levels (2.5 - 3 times control) were seen with an increase in $[K^+]_o$ of around 4 mM (Pentreath and Kai-Kai, 1982) which is considered to occur physiologically as a result of neuronal activity. These authors suggest that elevated $[K^+]_o$ acts as a neuron/glia signal to "provide a significant mechanism for ensuring continual energy supplies [for neurons] during and subsequent to intense activity". Indeed, a role for astrocytes as a "metabolic link" between blood stream and neurons in mammals has already been suggested (Chapter 1, see also Gray, 1961; 1964; Kuffler, 1967). Further, Phelps (1971) proposed that glucose transported from the blood might be stored as glycogen in astrocytes and subsequently released into the extracellular space for neuronal use, dependant on neuronal requirements, signalled to astrocytes by neurotransmitters.

Thus glycogen is a) found mainly in astrocytes in the CNS, b) rapidly turned over, c) controlled by enzymes which are known and relatively well understood, and d) affected by the activity of neurons in invertebrate preparations. Changes in astrocyte glycogen stores therefore seem to be a suitable response to study with respect to possible neuron/astrocyte signalling mechanisms.

The possible nature of the signal(s) between neurons and

astrocytes should now be considered. The signal(s) are presumably released from neurons. Tight junctions are not commonly seen between neurons and glia (Peters et al, 1976) so direct transfer seems unlikely. Neurons are known to release neurotransmitters which may act on the glycogen stores of astrocytes as was suggested by Phelps (1971) and as is discussed in Chapter 6. More fundamentally, it is known that neurons release K^+ from their axons and terminals during the course of action potential conduction and transmission (Frankenhauser and Hodgkin, 1956). Indeed it has already been noted that changes in $[K^+]_o$ can affect the glycogen stores of invertebrate glia. Furthermore, one of the major facts known about astrocytes in vivo is their responsiveness to K^+ as reflected in the dependance of their membrane potential to $[K^+]_o$.

In the mid 1950s it was reported that there were cells in the spinal cord (Coombs et al, 1955) and cerebral cortex (Phillips, 1956) which had large membrane resting potentials and did not show synaptic or action potentials when stimulated directly by electrodes, or indirectly by nerve volleys. These cells were termed "idle" or "silent" cells. The initial supposition that these were glial cells was later proved to be correct by various histological studies using intracellular dye injection into the "silent" cells after recording (see Orkand, 1978).

Further studies were then carried out in explant cultures of kitten CNS by Hild et al (1958; 1962) and by Kuffler, Nicholls and Orkand using the leech CNS and the Necturus (mudpuppy) retina and optic nerve (for review see Orkand, 1978). In these systems the neuroglial cells were comparatively easy to identify and to record from electrophysiologically when compared to astrocytes in situ in the mammalian brain. It was seen that glial cells generally have a transmembrane potential (Em) of between -70 and -90 mV (inside

negative) which is depolarised progressively as the $[K^+]_o$ increases. Detailed examination of the mud puppy optic nerve showed that the Em of the glial cells surrounding the axons of the optic nerve varied with $[K^+]_o$ exactly as predicted by the Nernst equation. This indicated that glial cells have a high degree of membrane permeability for this ion. Alterations in the concentrations of other ions such as Na^+, Cl^- and Ca^{2+} show that the K^+ permeability determines primarily this membrane potential (Orkand, 1978). The conclusion for the Necturus optic nerve glia has also been shown to be true for mammalian astrocytes in vivo, and in primary culture as reviewed by Walz and Hertz (1983).

As previously noted, the ionic fluxes across the neuronal membrane during an action potential result in a loss of K^+ from neurons and this release has been implicated as the cause of the slow depolarisation of cortical glial cell membranes during increased neuronal activity in the cortex (Karamashi and Goldring, 1966). In the cat cortex, K^+ sensitive electrodes have been used to demonstrate the correlation of intercellular K^+ levels with increased neuronal activity (Prince et al, 1973). This K^+ exchange may serve as a signalling mechanism at least in the neuron-to-astrocyte direction.

Thus a series of experiments was designed to examine the effects of $[K^+]_o$ on astrocyte glycogen stores with the aim of determining a) whether astrocytes in vitro contain glycogen and b) whether astrocytes respond to changes in $[K^+]_o$ using glycogen as an indicator.

Recent work from our laboratory (Pearce et al, 1985a,b) has shown that neonatal rat cortical astrocytes in vitro can incorporate [^{14}C]-2-DG into glycogen. However, the use of radio-

labelled 2-DG incorporation into glycogen as a measure of glycogen leads to difficulties of interpretation. This is because the increased incorporation of radiolabelled 2-DG into glycogen does not discriminate between a net increase in glycogen or an increase in turnover rate. It is therefore preferable to measure total glycogen stores. This can be achieved using the enzymatic breakdown of glycogen by amylo-α1,4 α1,6-glucosidase (EC.3.2.1.3) (AG) and subsequent measurement of the released glucose by hexokinase and glucose-6- phosphate dehydrogenase (Lust and Passoneau, 1975).

In this chapter it will be shown that:-

i) Glycogen is contained in cultures of astrocytes and meningeal cells.

ii) The glycogen levels of astrocyte cultures are affected by increases in $[K^+]_o$ of the order (2.5 mM - 12.5 mM) believed to occur in vivo as a result of neuronal activity.

iii) Meningeal cells in vitro also possess glycogen stores which are affected by increases in $[K^+]_o$. However, the time and concentration dependency of the effects on meningeal stores differ from that found in astrocyte cultures.

Methods

Cell cultures

Astrocyte and meningeal cultures were prepared as previously described (Chapter 3) and grown on 60 mm diameter plastic dishes. The cultures were used at confluence (18 - 22 d.i.v. for astrocytes; 11 - 15 d.i.v. for meningeal cells) with medium changes at 6 d.i.v. for astrocyte and meningeal cells, and at 13 d.i.v. for astrocytes only.

K^+ exposure

In order to produce maximal levels of glycogen in these cultures, the glucose concentration of the culture medium was increased to 20 mM two hours prior to the start of the experiment (Cummins et al, 1983). The culture medium was removed after the 2 hour pre-incubation period and the cultures washed with 3 mls of pre-warmed buffer (NaCl, 120 mM; $NaHCO_3$, 10 mM; $NaH_2PO_4.2H_2O$, 1 mM; KCl, 2.5 mM; D-glucose, 20 mM; $MgSO_4.7H_2O$; 1.5 mM; $CaCl_2.2H_2O$, 2.5 mM; HEPES, 20 mM; pH 7.4 with HCl; at 37°C); 5 mls of buffer was then added to the washed cultures. In experiments where the $[K^+]_o$ was raised, the KCl concentration was increased (the NaCl concentration was correspondingly reduced to maintain osmolarity) and the cultures washed with buffer containing the appropriate K^+ concentration. The cells were then incubated (35.5°C, 3% CO_2/air) for 10, 30, 60 or 90 minutes. Four different $[K^+]_o$ were used in each experiment, 2.5, 5, 10 and 15 mM. Typically, two cultures were used per concentration of $[K^+]_o$, totalling 8 cultures, and all cells were incubated for the same time period in any one experiment. At the end of the incubation period, the buffer was removed and the cells washed twice with 3 mls of ice cold 0.9% NaCl. 800 µl of 0.1 N NaOH was added to each dish and the cells harvested.

Statistical Analysis

It was noted that there was marked variability in the glycogen content of cell cultures between replicate experiments. In order to reduce the effect of this variation, the results were analysed by comparing the glycogen contents of experimental cultures to those of control cultures (incubated in 2.5mM $[K^+]_o$) from the same experiment. For example, the effect of incubating astrocyte cultures with experimental K^+ concentrations for 30 minutes was determined in three experiments with two cultures used per $[K^+]_o$ in each experiment. The paired data (i.e. experimental and 2.5mM control) from the three experiments were then pooled and analysed by matched pair 2 tailed t-test (Snedecor and Cochran, 1967). Typically, a significant difference was only found when all the values of experimental culture glycogen content were less than their corresponding control values.

Comparisons were also made between the glyocgen contents of cell cultures at different time points. As a complete time course was not performed in any one experiment, these data could not be matched and analysed as before. Instead the data were expressed as percentage values of their corresponding control value (at 2.5mM $[K^+]_o$ and analysed using Kruskal-Wallis non-parametric analysis of variance to determine whether there were statistically significant changes in cell culture glycogen with time.

The glycogen content of the cultures was determined using a method based on Passoneau and Lauderdale (1974), Lust et al (1975) and Cummins et al (1983a).

i) The cell suspension was transferred to 1.5 ml capped, conical Eppendorf tubes and the glucose in the suspension was degraded by heating at 80°C for 20 minutes (Lust et al, 1975).

ii) Aliquots were then taken for determination of protein by the method of Lowry et al. (1951), using BSA as a standard (Sigma, fraction V).

iii) Material insoluble in 0.1N NaOH was removed by centrifugation at 12,400 g for 10 minutes (Microfuge 12, Beckman Instruments).

iv) The supernatant was transferred to 5 ml conical tubes and 1.6 mls of 95% ethanol was added to the supernatant (final concentration approximately 66%) in order to precipitate the glycogen (Bernard, 1857; Pfluger, 1904).

v) The glycogen was pelleted by centrifugation at 3000 r.p.m. (1600 g) for 15 minutes in a Coolspin centrifuge (MSE Scientific Instruments).

vi) After removal of the supernatant the pellet was resuspended in 100 µl of 0.1M sodium acetate buffer (pH 4.7) and the glycogen reprecipitated and pelleted by the addition of 200 µl of 95% ethanol and centrifugation as in iv). The double precipitation of glycogen with ethanol was found to be necessary as glycogen could not be detected in aliquots of supernatant taken from step ii) as suggested by Cummins et al (1983a). A single precipitation step produced variable results in duplicates of control glycogen solutions dissolved in 0.1N NaOH (data not shown) and so a second precipitation step was used to wash out any remaining NaOH.

vii) The resulting pellet was resuspended in 100 µl of 0.1M sodium acetate buffer and degraded to glucose by the addition of 5 µl 20 mM Tris-HCl pH 7.5 + 0.02% BSA (Sigma fraction V) containing 5 µg of

of amylo-α1,4 - α1,6-glucosidase (AG; EC 3.2.1.3; Sigma grade IV from A. niger) and subsequent incubation at room temperature for 30 minutes (Passoneau and Lauderdale, 1974). The addition of 50 ng of AG as in the original method (Passoneau and Lauderdale, 1974) did not produce any measurable glucose from extracted glycogen standards. This may be due to the use of AG from Sigma rather than from Boehringer Mannheim GmbH which was used in the original method.

viii) The glucose thus produced was measured colorimetrically using a commercially available test kit (Hexokinase/glucose-6-phosphate dehydrogenase method; Boehringer Mannheim GmbH). The sensitivity of the assay meant that only one determination per culture was possible.

ix) Glycogen standards (rabbit liver; Sigma Type III, 1 - 10 µg in 0.1M sodium acetate buffer pH 4.7, in duplicate) were subjected to identical precipitation, enzymatic breakdown and glucose measurement procedures as described for glycogen derived from cell cultures. A standard curve for glycogen was prepared in each experiment.

x) In some cases, glycogen from cultures was not treated with AG in order to estimate the contamination of the purified glycogen with glucose, glucose-6-phosphate and other glucose containing compounds such as cerebrosides and gangliosides which affect the glucose assay.

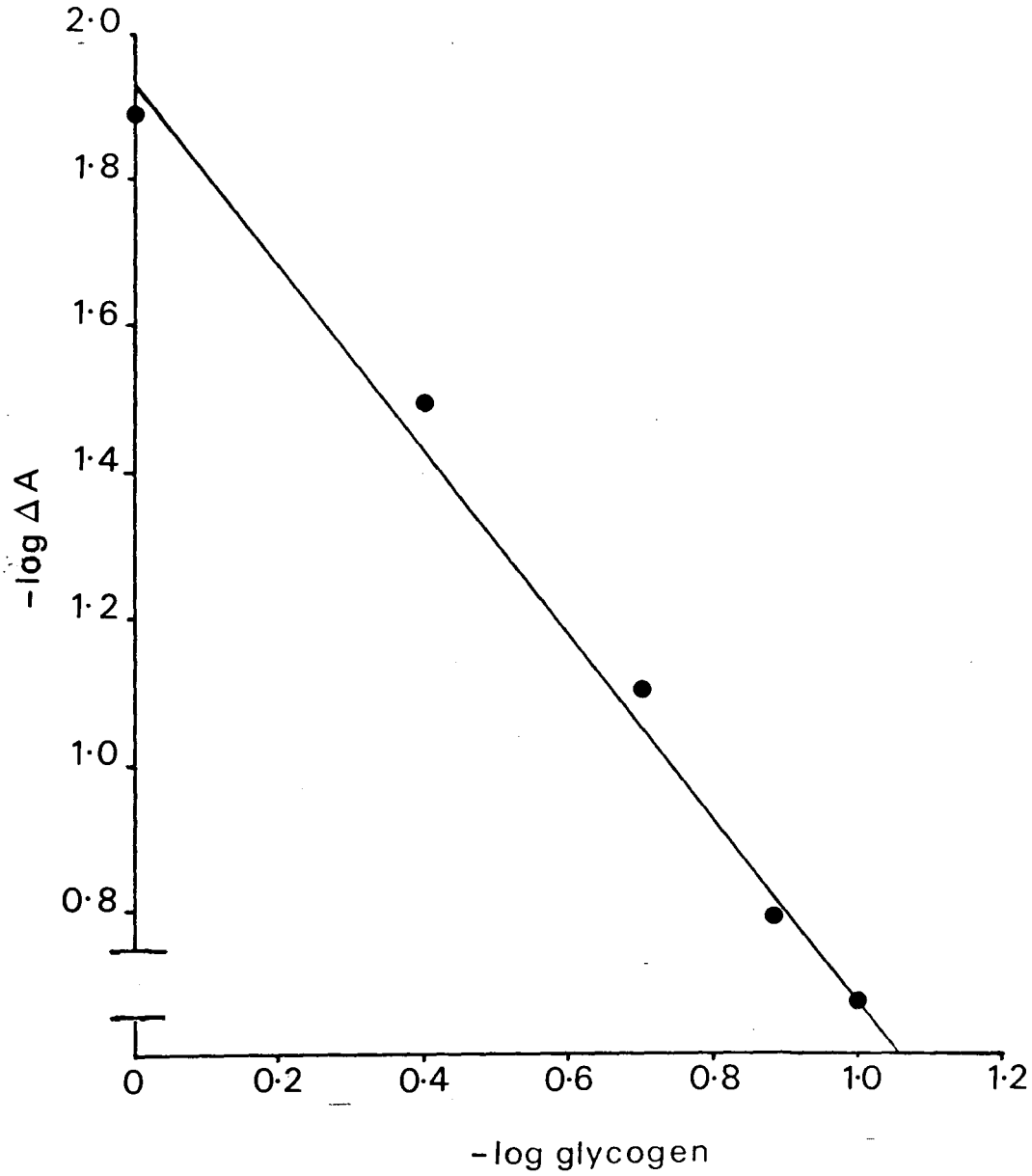
Results

Glycogen assay

Fig. 4.2 is a typical standard curve used for the estimation of glycogen content in cell cultures. It shows that there is a linear relationship between the log of the amount of standard glycogen before extraction (1 - 10 µg) and the log of the change in

Fig. 4.2

A typical standard curve for glycogen estimation


2·0
1·8
1·6
1·4
1·2
1·0
0·8
-log ΔA
0
0·2
0·4
0·6
0·8
1·0
1·2
-log glycogen

absorbance at 340 nM (ΔA) of the glucose assay mixture. The change in absorbance is caused by the production of NADPH from the action of hexokinase and glucose-6-phosphate dehydrogenase on glucose formed from the glycogen standards after extraction and enzymatic breakdown. This method permits measurement of amounts of glycogen as low as 0.5 μg per assay but not reproducibly as although the standard curve is linear, ΔA produced by a particular glycogen concentration varies between (but not within) extractions.

The modified method of glycogen extraction from cell cultures used here gives an extract which yields a range for ΔA in the glucose assay of 0.000 - 0.002 absorbance units (mean 0.001 ± 0.0004, n = 4) when pretreatment with AG is omitted. When the cell culture extracts were pretreated with AG however the values of ΔA were between 0.020 - 0.100 for astrocytes and between 0.010 - 0.300 for meningeal cells. This indicates that the extract is free of glucose and glucose-containing compounds measurable by the glucose assay used here.

Time course of glycogen accumulation in cell cultures

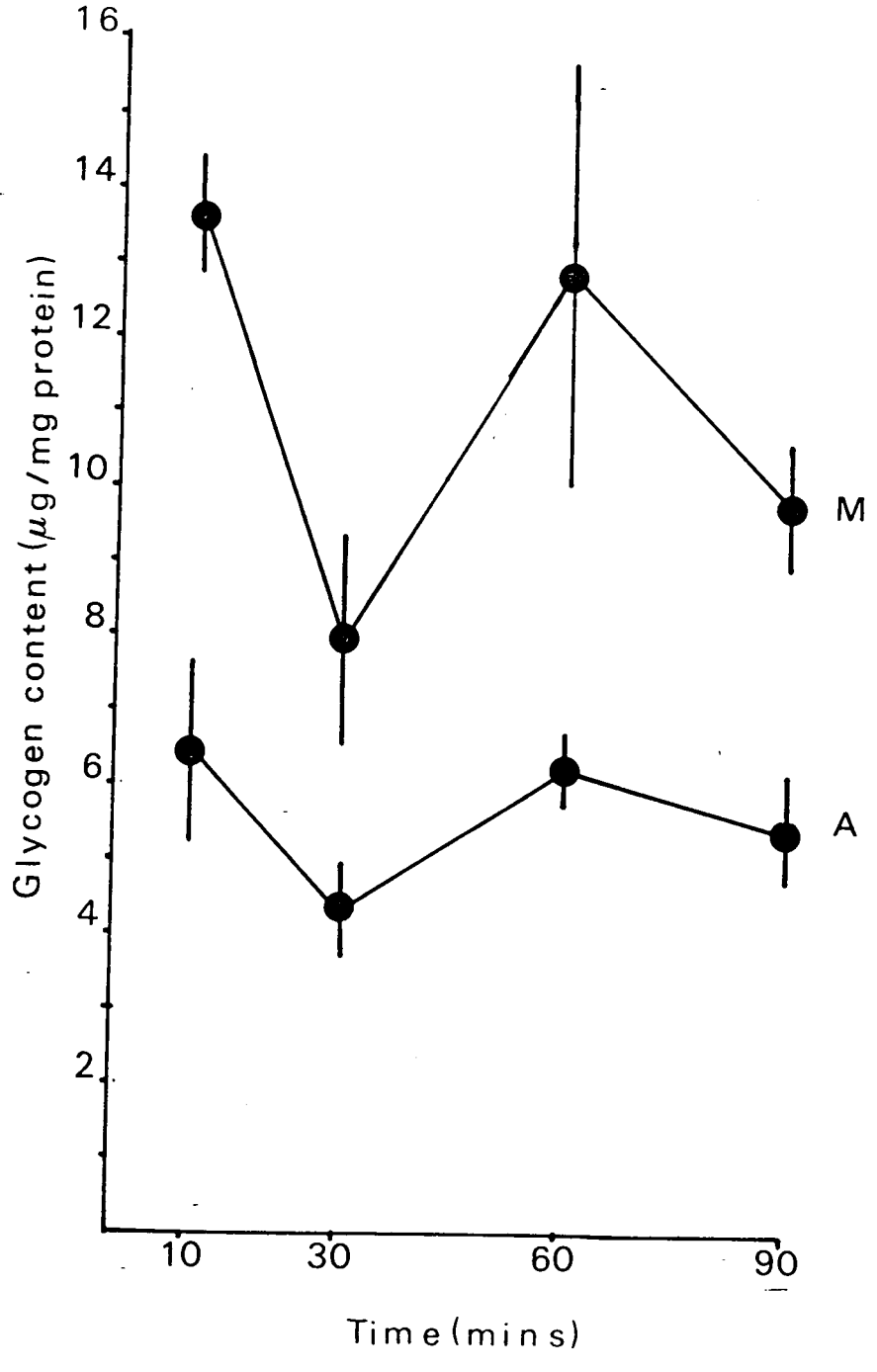
Fig. 4.3 represents the values for glycogen content in astrocyte and meningeal cell cultures incubated in 2.5 mM $[K^+]_o$ buffer for varying times (10, 30, 60 and 90 minutes). The results show a high degree of variability in the glycogen content of both cell types over time. It is evident, however, that there is no ongoing net synthesis of glycogen in either astrocyte or meningeal cell cultures over 90 minutes, as shown by one-way analysis of variance. It is also noticeable that the meningeal cell glycogen content is higher by approximately two times, than that in astrocyte cultures under control conditions

(2.5 mM $[K^+]_o$) at all time points.

Fig. 4.3

Time course of the glycogen content of astrocyte-enriched and meningeal cell cultures

Astrocyte-enriched (A) and meningeal cell (M) cultures were incubated in buffer containing 2.5 mM $[K^+]_o$ for various time intervals and the glycogen content determined as in Methods. The results shown are mean values with an n of between 6 - 13, where n is the number of individual dishes per time point. Bars indicate standard error of the mean.


16
14
12
10
8
6
4
2
Glycogen content (μg/mg protein)
M
A
10
30
60
90
Time (mins)

Effects of changes in $[K^+]_o$ on astrocyte glycogen stores

Fig. 4.4 shows the effect of increasing $[K^+]_o$ on astrocyte glycogen stores at four time points. The glycogen content of astrocyte cultures was statistically significantly less than control values at all time points, except 30 minutes, for 5mM $[K^+]_o$. At 10 minutes the reduction was 15%, 11% at 60 minutes and 10% at 90 minutes. Statistically significant reductions in glycogen stores are also seen at 90 minutes for 10mM $[K^+]_o$ (13%) and at 10 minutes for 15mM $[K^+]_o$ (30%). A significant increase in glycogen content is not seen under any conditions when compared against 2.5mM $[K^+]_o$ controls.

Kruskal-Wallis analysis of variance indicated that there were differences between the effects of experimental $[K_o]$ on astrocyte glyocgen content with time and indeed a consistent pattern is noticeable in the time course of the effects of all three experimental $[K^+]_o$. For all experimental $[K^+]_o$ there is a decrease in glycogen stores at 10 minutes when compared to control through this is not statistically significant for 10mM $[K^+]_o$. This initial decrease is reversed at 30 minutes for all experimental $[K^+]_o$ to levels not statistically significantly different from control. Indeed in the case of 15mM $[K^+]_o$ there is an increase in glycogen levels over control. By 60 minutes the glyocgen levels at all epxerimental $[K^+]_o$ have fallen again to show a decrease when compared to those at 30 minutes. In the case of 5mM $[K^+]_o$ the reduction in glycogen at 60 minutes is statistically significant when compared to control. At 90 minutes the glycogen content levels off in cultures incubated in 5 and 10mM $[K^+]_o$ and becomes statistically significantly less than control values with 5mM $[K^+]_o$ and 10mM $[K^+]_o$. The level of glycogen stored in astrocyte cultures at 90 minutes becomes progressively less in relation to control levels as the $[K^+]_o$ increases i.e. 10%, 13% and 19% at 5, 10 and 15mM $[K^+]_o$. The changes

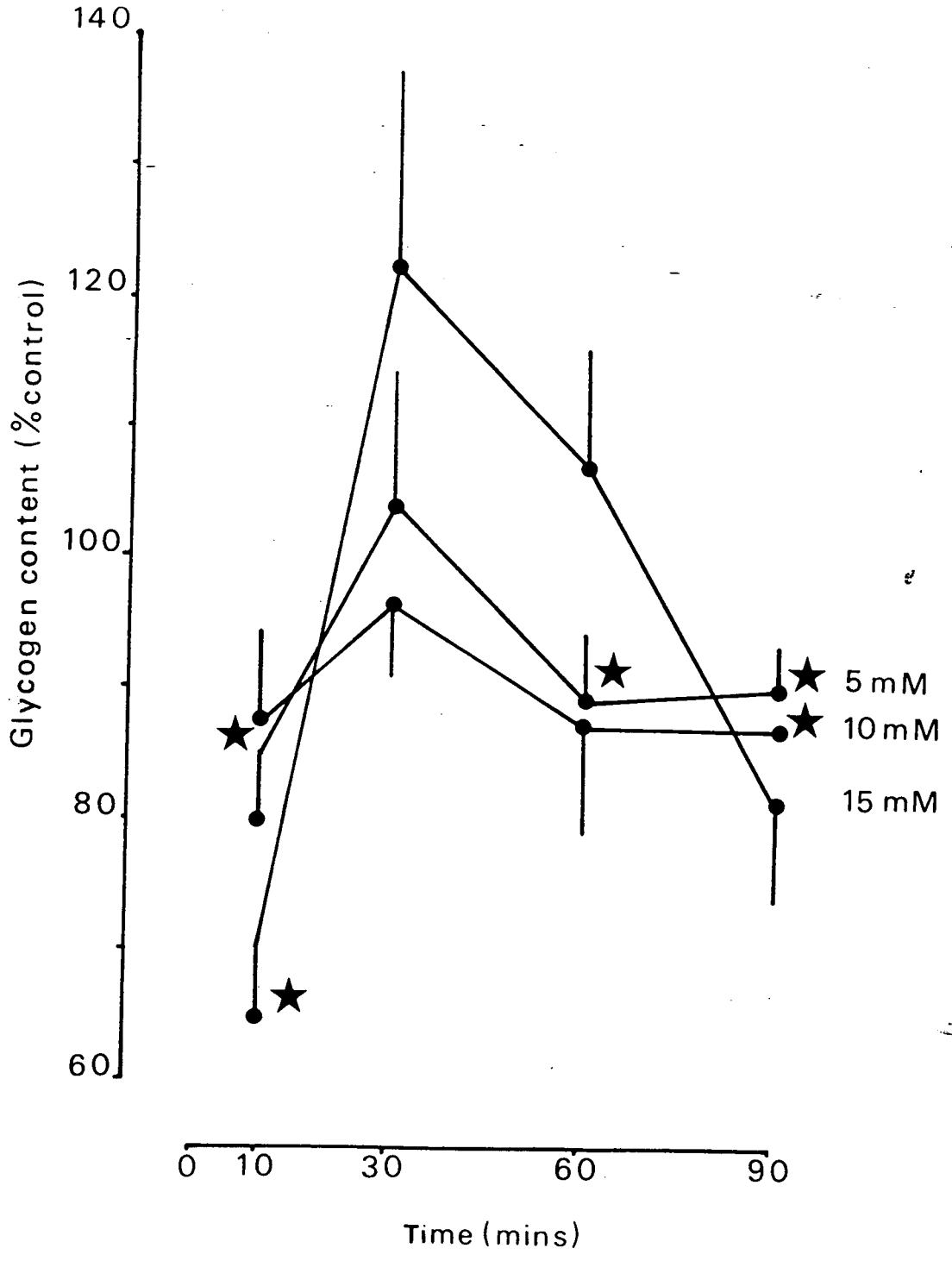
in glycogen content of astrocyte cultures over time suggest that after initial responses to increased $[K^+]_o$ involving both net synthesis and net degradation of glycogen, a new level of glycogen content is established which is dependant on $[K^+]_o$.

Fig. 4.4

The effect of changes in $[K^+]_o$ on astrocyte-enriched culture glycogen content

Fig. 4.4 shows the effect of elevated $[K^+]_o$ on the mean glycogen content of astrocyte cultures expressed as a percentage of control. Controls were taken as the glycogen content of cultures incubated for the same time, in the same experiments in 2.5 mM $[K^+]_o$. The results were obtained from between 2 - 5 experiments per time point and an n of between 6 - 11, where n is the total number of cultures that were compared against a mean value of glycogen content at 2.5 mM $[K^+]_o$ derived from 2 cultures per experiment. Bars indicate the standard error of the mean values; for graphical presentation one direction is usually shown.

* Indicates a significant difference from control of $p<0.05$ or less (matched pair, two-tailed t-test).


140
120
100
80
60
Glycogen content (%control)
0
10
30
60
90
Time (mins)
5 mM
10 mM
15 mM

Effects of changes in $[K^+]_o$ on meningeal cell glycogen

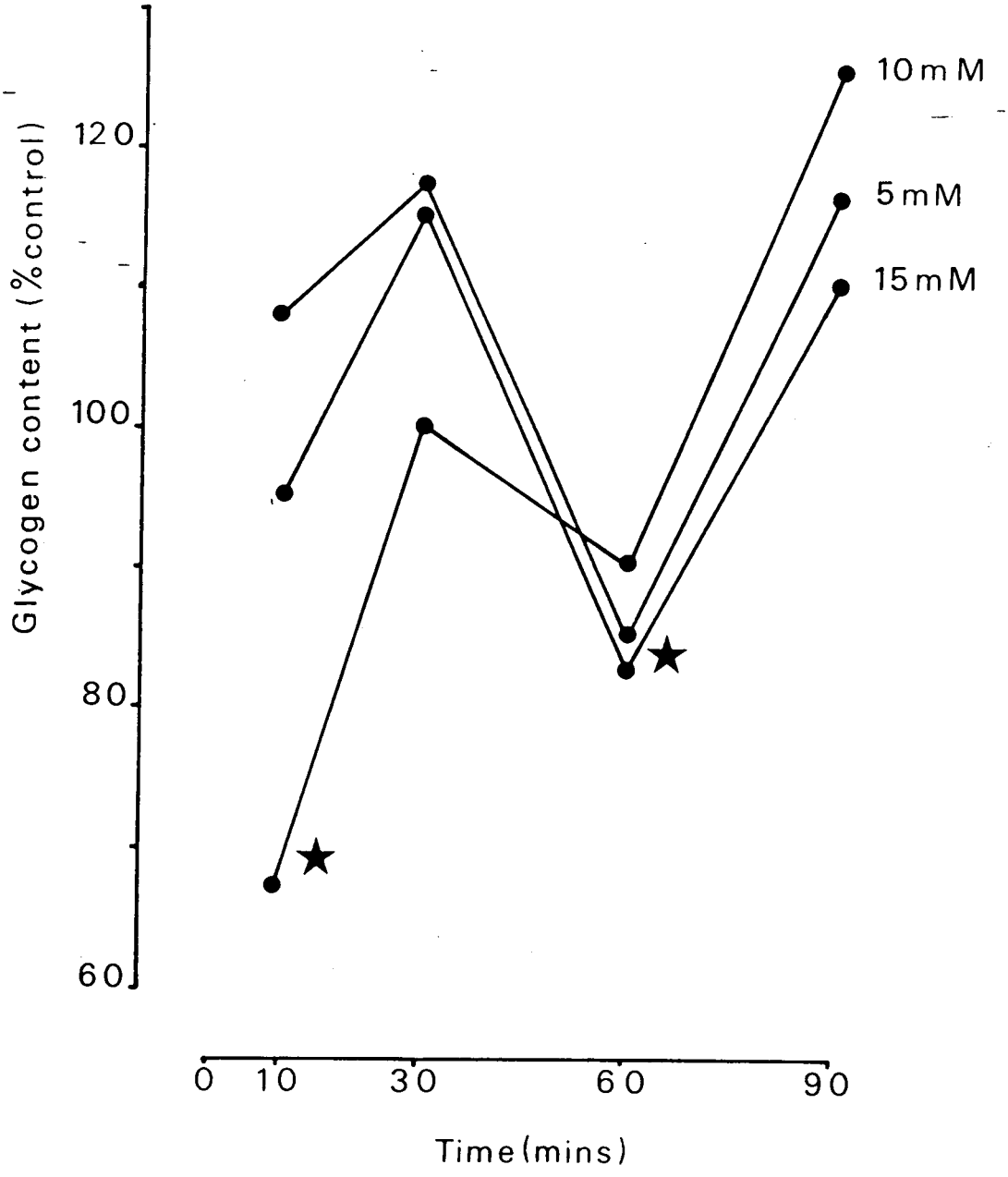
Fig. 4.5 shows the effect of increasing $[K^+]_o$ on the glycogen stores of meningeal cell cultures at four time points. Only two statistically significant changes in meningeal glycogen content are seen, at 10 minutes with 10mM $[K^+]_o$ and 60 minutes with 15mM $[K^+]_o$, showing decreases of 32% and 17% respectively. No statistically significant increases are seen in meningeal glycogen over controls when exposed to increased $[K^+]_o$. However, analysis of variance indicated that there were differences between the effects of experimental $[K^+]_o$ on meningeal cell glycogen content with time. As in the case with the effect of $[K^+]_o$ on astrocyte glycogen stores, there is a consistent pattern evident in the time course of the effects of all three experimental $[K^+]_o$ but this pattern is different from that described for astrocytes. There is an increase in levels of glycogen as compared to control values between 10 and 30 minutes for all experimental $[K^+]_o$ in meningeal cell cultures as there is in astrocyte cultures. This relative increase is also seen to diminish subsequently to levels below control values, in one case a statistically significant decrease (60 minutes at 15mM $[K^+]_o$) as is seen in astrocytes but a similar levelling off or further decrease which is observed at 90 minutes in astrocyte cultures is not apparent. Instead there is a second increase in glycogen stores to above that of control. The time course of the effects of increased $[K^+]_o$ on meningeal cell glycogen taken as a whole suggests that whilst both net synthesis and net degradation of glycogen occur in response to increased $[K^+]_o$, the responses are a) highly variable, and b) only statistically significant at higher levels of $[K^+]_o$ (10 and 15mM) than those seen to reduce consistently the glycogen content of astrocyte cultures (5mM $[K^+]_o$).

Fig. 4.5

The effect of changes in $[K^+]_o$ on meningeal cell culture glycogen content

This figure shows the effect of increasing $[K^+]_o$ on the mean glycogen content of meningeal cultures expressed as a percentage of the glycogen content in cultures incubated for the same time, in the same experiment in 2.5 mM $[K^+]_o$ as control. The results were obtained from 3 experiments per time point and an n of between 5 and 7 where n is the total number of individual cultures that were compared against a mean value of glycogen content at 2.5 mM $[K^+]_o$ derived from 2 cultures per experiment. Standard errors of the means varied between 5 - 18% and are not shown for reasons of clarity.

* Indicates a signficant difference from control of $p<0.05$ or less (matched pair, two-tailed, t-test).


Glycogen content (%control)
120
100
80
60
10mM
5mM
15mM
0
10
30
60
90
Time(mins)

Discussion

The major findings of this study are:-

i) Both astrocyte and meningeal cells in culture metabolise glycogen.

ii) There is no net synthesis or degradation of glycogen stores in astrocyte or meningeal cell cultures over the incubation period used.

iii) Changes in $[K^+]_o$ affect the glycogen stores of astrocytes and meningeal cells but the two cell types differ in their responses to $[K^+]_o$.

iv) These cultures have thus been shown to contain viable cells which respond to changes in their environment and as such constitute a good model to examine the effects of putative neuron/astrocyte signals controlling glycogen stores in astrocytes.

Prior to discussion of the $[K^+]_o$ evoked responses observed here and of whether or not the responses might form the basis of a neuron/astrocyte signalling mechanism, it is important to know the normal levels of $[K^+]_o$ in the CNS at rest and during neuronal activation.

Experiments with ion selective electrodes estimate the resting $[K^+]_o$ in the mammalian cortex at between 2.8 mM and 3.4 mM (Walz and Hertz, 1983 review), so a value of 2.5 mM $[K^+]_o$ in the experimental buffer might be used to estimate control levels of glycogen in cell cultures. Sensory stimulation produces increases in $[K^+]_o$ in the region of 0.1 - 1 mM in the mammalian CNS (Kelly and van Essen, 1974; Somjen, 1979) and direct stimulation of the cat cortex does not increase $[K^+]_o$ beyond 12 mM (Grossman et al, 1969; Prince et al, 1973). Only during the abnormal conditions of seizures, spreading depression and hypoxia do $[K^+]_o$ levels rise above 10 - 12 mM; 13 mM in seizures and between 30 mM and 100 mM in spreading depression and hypoxia

(Walz and Hertz, 1983). The use of 10 mM and 15 mM $[K^+]_o$ in experiments designed to examine a proposed neuron/astrocyte signalling mechanism through K^+ release from neurons undergoing the normal depolarisations involved in action potential conduction and transmission, may therefore seem irrelevant. However, the increases in $[K^+]_o$ measured in in vivo experiments generally employ "ion selective" electrodes which respond to a range of compounds other than K^+ (Wuttke and Schlue, 1982). Furthermore, an electrode creates a dead space around the tip which may reduce the estimated size of a transient increase in $[K^+]_o$ due to the resultant delay in measuring an ion which is both actively and passively removed from the extracellular space both by neurons and astrocytes (Walz and Hertz, 1983). The possibility that $[K^+]_o$ may routinely reach 15 mM locally cannot be entirely excluded, although experiments which estimate $[K^+]_o$ by measuring glial depolarisation and assuming that this is a perfect K^+ electrode system agree that direct stimulation never raises $[K^+]_o$ beyond 12 mM (Kelly and van Essen, 1974).

Previous studies on the effect of K^+ on cerebral glycogen stores have generally been confined to experiments using relatively high $[K^+]_o$ (in the region of 25 - 50 mM) and cortical slices, or pathological conditions such as ischaemia and seizures, and are therefore of limited value when considering physiologically relevant neuron/astrocyte signals. However, these studies suggest that increased levels of $[K^+]_o$ may favour a net reduction in glycogen stores.

For example, Quach et al (1978), who examined the effect of elevated $[K^+]_o$ on glycogen stores in mouse cortical slices, showed an 87% reduction in glycogen synthesised from radiolabelled glucose. However, the stimulus was 50 mM $[K^+]_o$ which might have elicited the

release of a wide range of neurotransmitters and these may have effects on glycogen stores (Chapter 6). Magistretti et al (1983) also showed that increases in $[K^+]_o$ could evoke the breakdown of radiolabelled glycogen (30%), in this case using rat astrocytes in primary culture, but only examined the effect of 50 mM $[K^+]_o$.

Therefore, there are few published data concerning the effects of changes in $[K^+]_o$ on astrocyte glycogen stores. Furthermore, only the work of Pentreath and co-workers (see Introduction) has examined the effects of increased $[K^+]_o$ on glial glycogen stores with respect to the concept of neuron/glia signalling. This is also true when other aspects of glial metabolism are considered. It is well known that high levels of $[K^+]_o$ have profound effects on glial cell metabolism e.g. a reduction in ATP levels and an activation of Na^+/K^+ ATPase (see review by Hertz and Schousboe, 1975). In particular it has often been shown that K^+ stimulates oxygen uptake in bulk prepared glia (Haljamae and Hertz, 1971) and in cultured astrocytes (Hertz et al, 1973). However, these experiments again use particularly high levels of $[K^+]_o$, in the region of 50 mM, and there are few data on the effects of changes in $[K^+]_o$ more commonly found in the mammalian CNS. For example, Orkand et al (1981) have shown that changes in $[K^+]_o$ in the range 3 - 9 mM produce an increase in the oxidation of NADH to NAD^+, and an increased uptake and incorporation of radiolabelled glucose into organic and amino acids in the denervated Necturus optic nerve. In addition, Cummins et al (1975) observed that an increase in $[K^+]_o$ from 7 mM to 12 mM caused an increase in $[^3H]$-2-DG uptake and a decrease in methionine uptake into primary cultures of rat astrocytes.

When the data presented here are considered, it is seen that the

glycogen content of astrocyte cultures prepared as previously described (Chapter 3 and Methods) is lower than had previously been reported for rat astrocytes in vitro. Cummins et al (1983a) measured the glycogen content of neonatal rat primary cultures of astrocytes, together with the activities of GS and GP, subsequent to treating the cultures with glucose. Glycogen content was measured essentially as described here, i.e. by enzymatic breakdown to glucose which was measured by the hexokinase/glucose-6-phosphate dehydrogenase method. However, Cummins et al (1983a) measured glycogen in unextracted aliquots of cell suspension which was not possible here (see Methods). The reason for this difference is unclear. It may reflect the more frequent medium changes used by Cummins et al (1983a, every two days) or a greater number of cells in their cultures. Alternatively, it may be due to an increased meningeal cell contamination in their cultures, suggested by the fact that their cultures reach confluence more quickly (12 - 14 d.i.v.). The glycogen content of adult mammalian brain is approximately 30% of that perinatally (Kohle and Vanucci, 1977). This developmental loss in glycogen stores may be maintained in vitro in that older cultures such as those used here (18 - 22 d.i.v.), show a relative reduction in glycogen levels compared to younger cultures. However, it was possible to measure glycogen in the astrocyte cultures used here by concentrating it using ethanol precipitation.

Another point which is very clear from the results of early experiments measuring glycogen content of cell cultures is that meningeal cells contain almost twice as much glycogen per mg protein as astrocytes. Taking the value of 15% FN^+ cell contamination of astrocyte cultures (Chapter 3) to represent 15% meningeal cells, then, simplistically, it might be estimated that about 30% of the

glycogen in astrocyte cultures is found in meningeal cells. Bearing this point in mind it is seen that neither astrocytes nor meningeal cells show a net synthesis or degradation of glycogen stores over the experimental period (10 - 90 minutes). Indeed this provided a base line for further experiments on the glycogen content of astrocytes (Chapter 6). However, when the effects of increasing $[K^+]_o$ on cell culture glycogen stores are determined it is clear that in this respect astrocytes and meningeal cells respond quite differently. This is exemplified by the fact that the statistically significant differences from control in the glycogen content of the two types of cell culture when exposed to increased $[K^+]_o$ show no overlap. Thus the only statistically significant differences from control values for the glycogen content of meningeal cells are caused by $[K^+]_o$ of 10 mM (10 minutes) and 15 mM (60 minutes). At these points, astrocyte glycogen levels are not significantly different from control. These comparisons suggest that meningeal cell glycogen is not the only, or even principal, store of glycogen in astrocyte cultures. However, the possible contribution of meningeal cell glycogen to the total pool of glycogen in astrocyte enriched cultures cannot as yet be discounted. For example, if meningeal cells do contribute around 30% of the glycogen in the astrocyte culture glycogen pool then one might suggest that the non-statistically significant reduction in glycogen levels of astrocyte cultures at 10 minutes with 10 mM $[K^+]_o$ (13%) results from a 32% drop in meningeal cell glycogen. In spite of this, it is clear that $[K^+]_o$ evoked changes in meningeal cell glycogen do not account for those in astrocyte cultures. Moreover it is quite probable that meningeal cell glycogen, as a relatively K^+ unresponsive and variable pool (Fig. 4.4, Chapter 3), serves not only to contribute to the variability seen in astrocyte enriched culture stores of glycogen but also to dilute the observed effects of

$[K^+]_o$ on astrocyte stores. Unfortunately, as nothing is known about the glycogen stores of meningeal cells present in astrocyte cultures, it is impossible to correct for this eventuality. This will be discussed again later (Chapter 6) where it is suggested that meningeal cell glycogen contributes little to the total glycogen pool in astrocyte cultures as evidenced by the differential effects of adrenergic agonists on the glycogen stores of both types of cell culture.

Whilst the responses of the glycogen stores of astrocytes and meningeal cell cultures to increases in $[K^+]_o$ are statistically significant at different incubation times and $[K^+]_o$, there are similarities in the time course of these responses. In astrocyte cultures, and to a more varied degree in meningeal cell cultures, the first response to increased $[K^+]_o$ measured (at 10 minutes) is a reduction in glycogen stores compared to control. Thus 5 mM and 15 mM $[K^+]_o$ evoke statistically significant decreases in astrocyte cultures as does 10 mM $[K^+]_o$ in meningeal cell cultures. After this initial reduction, there is a period of resynthesis of glycogen between 10 - 30 minutes. This may restore levels of glycogen to control levels or above at 30 minutes prior to a subsequent relative loss of glycogen seen at 60 minutes in both cell cultures. In the case of astrocyte glycogen stores a new base line of glycogen content, below control, may now be established e.g. with 5 and 10 mM $[K^+]_o$, or the glycogen content may continue to fall in relation to control (15 mM $[K^+]_o$). In contrast meningeal cell glycogen stores undergo a second phase of net glycogen synthesis compared to control, such that at 90 minutes all experimental cultures contain more glycogen on average than control cultures. It must be stressed again that the $[K^+]_o$ evoked changes in meningeal cell culture glycogen content are rarely statistically significant but the contrast to the responses

of astrocyte cultures to increased $[K^+]_o$ at this time is marked.

It is notable that the most consistent (with time) reductions in astrocyte glycogen content occur as a result of relatively small changes in $[K^+]_o$ (2.5 - 5 mM) which are of the order known to occur in vivo as a consequence of neuronal activity. In contrast meningeal cell glycogen stores only respond to larger increases in $[K^+]_o$ (2.5 - 10 and 15 mM). It is also notable that the time course of the effects of increased $[K^+]_o$ on astrocyte glycogen content suggests that after a period during which processes of both net synthesis and degradation occur, a new level of glycogen content is established below that of control. Furthermore, the level to which the glycogen content of astrocyte cultures falls in relation to control values at 90 minutes becomes greater at higher $[K^+]_o$ (10%, 13% and 19% reductions at 5, 10 and 15 mM $[K^+]_o$ respectively).

The observation that increases in $[K^+]_o$ produce a lower level of glycogen content in astrocyte cultures is consistent with the model of Phelps (1971) who suggested that the amount of glycogen stored in astrocytes is dependant on the activity (and thus energy requirements) of neurons. The model proposed by Pentreath (1982), from work using $[^3H]$-2-DG incorporation into the glycogen stores of invertebrate glia, also suggests that glial glycogen may be affected by the activity of neurons. However, in this case, under conditions where glucose supply is optimal, K^+ released from active neurons is proposed to stimulate the synthesis of glycogen in glial cells as a reserve energy store for neurons. Indeed, in the liver, glycogen synthesis is favoured by high levels of K^+ (cf. Pentreath and Kai-Kai, 1982). Moreover, increases in $[K^+]_o$ (to 5 and 15 mM but not

to 10 mM $[K^+]_o$) are seen to stimulate the incorporation over one hour of [^{14}C]-2-DG into the glycogen stores (37% and 44% respectively) of similarly prepared astrocyte cultures to those used here (Pearce et al, unpublished observations). Before considering the implications of this apparent contradiction, certain points need to be made about the use of radiolabelled 2-DG.

Originally developed by Sokoloff et al (1977) for the estimation and mapping of energy utilisation in the CNS, 2-DG is taken up and phosphorylated by hexokinase in an identical manner to glucose, forming 2-deoxyglucose-6-phosphate (2-DG-6-P) (Sols and Crane, 1954). 2-DG-6-P is not a substrate for glucose-6-phosphate dehydrogenase and therefore cannot undergo glycolysis or be metabolised by the pentose phosphate shunt (Pentreath et al, 1982). However, it is metabolised by glucose-6-phosphatase in the brain (Karnosvsky et al, 1980), an enzyme present in astrocyte cultures (1.23 ± 0.04 µmol G-6-P/mg protein/hour n=3, Grimble unpublished observations). Also, as previously noted [^{3}H]-2-DG can be incorporated into glycogen where it is assumed to be trapped (Pentreath et al, 1982). Some of the 2-DG-6-P is probably reconverted to 2-DG which may help to explain why only 2 - 10% of added 2-DG is incorporated when glycogen has a high rate of turnover in the brain (Ibrahim, 1975) and in primary culture (Cummins et al 1983a). The statement that "2-DG is trapped in glycogen" means that there are only four enzymes of glycogen metabolism which can have an influence on its presence in glycogen. These are UDP-glucose pyrophosphorylase, phosphoglucomutase, glycogen synthetase and the branching enzyme, i.e. the enzymes involved in synthesis.

The time course results for the effects of both 5 mM and 15 mM

$[K^+]_o$ on astrocyte glycogen stores show a marked net resynthesis of glycogen between 10 and 30 minutes which could produce an increase in the incorporation of [^{14}C]-2-DG into glycogen. Notably, the time course for the effects of 10 mM $[K^+]_o$ on astrocyte glycogen shows a less marked period of resynthesis between 10 and 30 minutes which may account for the lack of increased [^{14}C]-2-DG incorporation into glycogen seen with this concentration. Thus the [^{14}C]-2-DG results of Pearce et al are in agreement with the measurements of total glycogen presented here and suggest that part of the response to increased $[K^+]_o$ is an increased synthesis of glycogen. This agreement depends on 2-DG being trapped in glycogen as asserted by Pentreath (1982). If 2-DG were not trapped in glycogen, but was removed by glycogen phosphorylase and/or the debranching enzyme in the same way as glucose, then the [^{14}C]-2-DG incorporation data should mirror values for total glycogen stores.

The trapping of 2-DG in glycogen is equivocal as freeze-blowing techniques (Veech and Hawkins, 1974) show that over 60% of [^{3}H]-2-DG incorporated into glycogen in adult rat brain is released during decapitation and dissection followed by freezing in liquid N_2 (Nelson et al, 1984). Although it is known that there is a rapid activation of GPa post-mortem (Ibrahim, 1975), the exact mechanisms involved in the post-morten release of 2-DG from pre-labelled glycogen in whole brain are not known and may not be active in the astrocyte cultures used here. Indeed it would be difficult to reconcile the results shown here if [^{14}C]-2-DG were not trapped in the glycogen stores of astrocyte cultures under the experimental conditions previously described.

Prior to further discussion of the data presented here, the non-

physiological aspects of the study of glycogen metabolism in the cell cultures used should be noted.

Firstly, the exposure of astrocytes to prolonged increases in $[K^+]_o$ is unlikely to occur in vivo although it will depend on the substained activity of the neurons and the proximity of the astrocytes to the neurons. K^+ is actively and passively removed from the extracellular space by uptake primarily into glia (Walz and Hertz, 1983) as a mechanism of $[K^+]_o$ homeostatis. Therefore greater emphasis might be placed on the initial glycogenolytic effects caused by $[K^+]_o$ in the astrocyte cultures.

Secondly, passive uptake of K^+ by spatial buffering does not occur in primary cultures due to the uniform exposure of the cell to K^+ (Walz and Hertz, 1983). Bulk prepared adult astrocytes respond to increases in $[K^+]_o$ by an increase in the active uptake of K^+ via the Na^+/K^+ ATPase system (Franck et al, 1983). If this stimulation of an active uptake process is also present in the primary astrocyte cultures used in the present study then it might be argued that the initial period of glycogenolysis seen in astrocyte cultures is the result of the mobilisation of glycogen to provide energy for this uptake. As glucose is freely available in the experimental buffer used for these studies this is considered unlikely.

Finally, the astrocyte and meningeal cell cultures used here have been grown in a medium containing approximately 5 mM K^+ for 2 - 3 weeks as this is not optimal for neuronal survival (Chapter 3). The data previously discussed for the glycogen content in astrocyte and meningeal cell cultures under all conditions of $[K^+]_o$ might therefore be interpreted differently, using 5 mM $[K^+]_o$ as a control

value for estimation of glycogen content. As the astrocyte membrane is selectively permeable to K^+ it will act as a K^+ electrode and show a relative hyperpolarisation when the $[K^+]_o$ is changed from 5 mM in culture medium to 2.5 mM under experimental conditions. Hyperpolarisation of glial membranes also occurs after the termination of direct cortical stimulation (Grossman and Rosman, 1971; Ransom and Goldring, 1973a).

If the results are expressed as % values for glycogen content of cultures exposed to 5 mM $[K^+]_o$ as control, then a decrease in $[K^+]_o$ to 2.5 mM results in statistically significant increases in glycogen stores in astrocytes at 10, 60 and 90 minutes (15%, 11%, 10% respectively). The increases in stores of glycogen do not appear to involve an increase in glycogen synthesis as the incorporation of $[^{14}C]$-2-DG over 60 minutes is less in 2.5 mM $[K^+]_o$ than in 5 mM $[K^+]_o$ as previously noted. However, the apparent increase in astrocyte glycogen stores could be accounted for by a decrease in GPa activity in cells exposed to 2.5 mM $[K^+]_o$ compared to GPa activity in those cells exposed to 5 mM $[K^+]_o$. To explain the higher incorporation of $[^{14}C]$-2-DG into astrocyte glycogen stores at 5 mM $[K^+]_o$ compared to that at 2.5 mM $[K^+]_o$ after 60 minutes when the net glycogen content is greater in cultures exposed to 2.5 mM $[K^+]_o$, a higher activity of both GSa and GPa must occur in 5 mM $[K^+]_o$ treated cells i.e. a greater turnover rate. Such a mechanism whereby a hyperpolarisation of glial membranes promotes glycogen synthesis by reduced GPa activity (an "off" signal from neurons to astrocytes) is not mutually exclusive with an alternative interpretation of the present results that a depolarisation caused by rising $[K^+]_o$ (an "on" signal from neurons) evokes glycogenolysis.

The intracellular mechanisms through which $[K^+]_o$ might affect glycogen metabolism in cell cultures are unclear. Ververken et al (1982) noted that the activation of GPa observed in mouse cortical slices caused by incubation with 25 mM $[K^+]_o$ was not accompanied by any rise of cAMP level. The degree of K^+ stimulated activation of GPa was reduced by the addition of the Ca^{2+} chelating agent EGTA and also $LaCl_3$, to the experimental buffer. This suggests that the stimulation of GPa activity caused by 25 mM $[K^+]_o$ is mediated by a cAMP independant, Ca^{2+} dependant process. Quach et al (1978) suggest that 50 mM $[K^+]_o$ may also elicit glycogenolysis of glycogen pre-labelled with [^{3}H]-glucose in a cAMP independant manner. In addition, although not examining the effects of $[K^+]_o$, Passoneau and Crites (1976), in experiments to determine the mechanisms of regulation of glycogen metabolism in C6 cells, suggest that the control of glycogen metabolism by glucose is cAMP independant. These results suggest that there may be a level of control of glycogen metabolism in the CNS, particularly glycogenolysis, which is not linked directly to cAMP. No information is available concerning the mechanism(s) by which increases in $[K^+]_o$ might evoke the increases in glycogen synthesis reported here.

Therefore, the exact mechanism(s) by which increases in $[K^+]_o$ may affect the glycogen metabolism of astrocytes is unclear. Indeed, the direction of the effect, i.e. an increase or decrease in glycogen stores, depends to some extent on what is regarded as a control level of $[K^+]_o$. However, it is evident that alterations in $[K^+]_o$ over the accepted physiological range (2.5 mM - 5 mM $[K^+]_o$) influence the net glycogen stores found in astrocyte cultures.

Two models have previously been proposed (Phelps, 1971; Pentreath, 1982) which suggest that neurons control the metabolism of glycogen in astrocytes in order to ensure a continued supply of energy for neuronal activity. The results shown here provide additional evidence for this idea. In addition, a role for meningeal cells is proposed. In vivo meningeal cells, and other cells of the CNS vascular system, are interposed between blood and astrocytes. Isolated rat brain microvessels take up glucose through a facilitated transport mechanism (Djuricic, 1983) and meningeal cells synthesise glycogen in vivo (Ibrahim, 1975) and in vitro (this chapter). Statistically significant net reductions in meningeal glycogen stores are seen to be caused by increases in $[K^+]_o$ (10 and 15 mM) although these increases are greater than those which first produce effects on astrocyte glycogen stores (from 2.5 to 5 mM). This may indicate that the glycogen reserves are utilised only when $[K^+]_o$ reach pathological levels (see Introduction) and may constitute an emergency reserve of energy for neurons. However, little is known of the $[K^+]_o$ to which meningeal cells are normally exposed.

The inherent assumption of the models previously discussed is that there is a transfer of glucose or other metabolic substrates from astrocytes to neurons which is controlled by neurons. In this chapter, the role of one putative signal has been investigated $[K^+]_o$. Before considering this postulated transfer more fully (Chapter 7), the role of other possible signals between neurons and astrocytes, namely neurotransmitters, will be examined (Chapters 5 and 6).

Summary

1) Astrocytes and meningeal cells in primary cultures contain glycogen which they metabolise as shown by measurements of glycogen stores.

2) Both astrocyte and meningeal cell cultures respond to changes in $[K^+]_o$ by alterations in glycogen metabolism.

3) The responses to $[K^+]_o$ by astrocyte and meningeal cell cultures are different. Astrocytes in culture respond to smaller changes in $[K^+]_o$ than do meningeal cell cultures.

CHAPTER 5

Neurotransmitter receptors on astrocytes

Introduction

It has been shown that rat cortical astrocytes in vitro respond to variations in $[K^+]_o$ with changes in their metabolism of glycogen. In addition, it has been suggested that changes in $[K^+]_o$ might represent one means by which neuronal activity could be signalled to astrocytes in vivo, the result of which might be to increase supplies of metabolic substrates for neurons. Further, it has been suggested that neurotransmitters released from neurons might also serve as signals from neurons to astrocytes. This latter suggestion implies that astrocytes possess receptors for neurotransmitters on their membranes. To seek evidence for such a suggestion, this chapter describes experiments designed to examine the binding of three radiolabelled neurotransmitter receptor ligands to membranes prepared from astrocyte-enriched and meningeal cell cultures of newborn rat cortex. The three transmitter receptor ligands are [^{3}H]- dihydroalprenolol ([^{3}H]- DHA; β-adrenergic receptor antagonist), [^{3}H]-serotonin (serotinergic receptor agonist) and [^{3}H]- quinuclidinyl benzilate ([^{3}H]- QNB; muscarinic cholinergic receptor antagonist). Receptors for the first two have been implicated in the mediation of neurotransmitter-induced glycogenolysis in a variety of systems (Chapter 6); stimulation of the muscarinic cholinergic receptor has not been reported to have effects on glycogen stores in the brain.

Reports of neurotransmitter receptors on astrocytes whether in

Table 5.1.

A survey of the receptors for neurotransmitters which have been reported to be present on astrocytes.

Table 5.1 Astrocyte neurotransmitter receptors

Receptor	System	Technique	References
β-adrenergic (β_1 and β_2)	Chick & Rodent primary culture Rodent explant culture C6 glioma Human astrocytoma	Electrophysiology Ligand Binding cAMP assay Autoradiography Taurine release	Hosli et al (1982a,b) Shain & Martin (1984) Maderspach & Fajszi (1983) Trimmer et al (1984) Hansson et al (1984)
α-adrenergic (α_1 and α_2)	Rodent primary culture Rodent explant culture C6 glioma	Electrophysiology Autoradiography Ligand Binding cAMP assay Inositol phspholipid metabolism	Hosli et al (1982a,b) McCarthy & de Vellis (1979) Ebersolt et al (1981a) Pearce et al, (1985c)
Serotonin	Rodent primary culture Bulk separation of horse striatum C6 glioma	Ligand binding cAMP assay Ca^{2+} influx Inositol phospholipid metabolism	Fillion et al (1983) Tardy et al (1982) Sugino et al (1984) Bottenstein & de Vellis (1978) Pearce et al (1985c)
Dopamine	Bulk separation of rat CNS, bovine candate, horse striatum Rodent primary culture	cAMP assay	Palmer (1973) Henn et al (1980) Fillion et al (1980) Hansson et al (1984)
Histamine (H1 and H2)	Rodent primary culture Rodent explant culture Human astrocytoma	cAMP assay Electrophysiology Autoradiography	Clark et al (1975) Ebersolt et al (1981c) Hosli et al (1984) Hosli and Hosli (1984)
Adenosine (A1 and A2)	Rodent primary culture Chick primary cutlure Human astrocytoma	Ligand binding cAMP assay	Clarke et al (1975) Van Calker & Hamprecht (1980) Barnes & Thampy (1982) Ebersolt et al (1981c)
Muscarinic cholinergic	Chick primary culture Human astrocytoma C6 glioma Rodent primary culture	Ligand binding Electrophysiology cAMP assay Inositol phospholipid metabolism	Repke & Maderspach (1982) McCarthy & Harden (1982) Hamprecht et al (1976) Bottenstein & de Vellis (1978) Pearce et al (1985c)
Benzodiazepines	Rodent primary culture Bulk separation of bovine cortex and caudate	Ligand binding	Henn et al (1980) Bender & Hertz (1984) Sher & Machen (1984)
Peptides (VIP, MSH, SP, SOM, glucagon, ACTH, parathyrin, clacitonin, morphine, enkephalin)	Rodent primary culture Chick primary culture Human astrocytoma	cAMP assay [^{14}C]-2-DG incorporation	Evans et al (1984) Loffler et al (1982) Koh et al (1984) Rougon et al (1983) Pearce et al (1985b)
Prostaglandins	C6 glioma Rodent primary culture	cAMP assay	Clark et al (1975) Evans et al (1984)
Amino-acids (Glutamate, aspartate, GABA)	Rodent primary culture	Electrophysiology	Bowman & Kimelberg (1984) Kettenmann et al (1984)

primary culture, bulk separated adult glia or transformed cell lines, are numerous (Table 5.1). Some evidence has come from radiolabelled ligand binding studies, for example the binding of [^{3}H]- diazepam to bulk separated astrocytes from the bovine cortex (Henn and Henke, 1978). However, the bulk of the evidence is derived by inference from studies which examined the effects of transmitter agonists on astrocyte cyclic nucleotide levels often, particularly in early work, using C6 glioma (see Van Calker and Hamprecht, 1980, for review, also Table 5.1).

Since the review of Van Calker and Hamprecht (1980) the use of better defined astrocyte culture preparations and techniques of immunofluorescence, autoradiography and electrophysiology have provided additional evidence for some, but not all, neurotransmitter receptors on astrocytes. The most convincing demonstration of this comes from the work of McCarthy and co-workers (Harden and McCarthy, 1982; McCarthy, 1983; Trimmer et al, 1984) who have shown by combined autoradiography and immunofluorescence that approximately 85% of astrocytes in vitro prepared from neonatal rat cortex possess β-adrenergic receptors. Moreover, by using highly purified astrocyte cultures (>97%) they have shown that this receptor is of the β_1 subtype. Additional evidence for the presence of β-receptors on astrocytes comes from the autoradiographic and electrophysiological studies of Hosli et al (1982a; 1982b) using explant culutres of the foetal and neonatal rat brain stem, cerebellum and spinal cord. In the same system these authors have also shown the presence of α_1 and α_2 adrenoreceptors on astrocytes, again by autoradiography and electrophysiology, in partial agreement with the electrophysiological studies of Hirata et al (1982) who did not differentiate between the two α-receptor subtypes.

Interestingly, stimulation of α and β-receptors produces different effects on the membrane potential of astrocytes in vitro. α- receptors seem to mediate the noradrenaline evoked depolarisation of astrocyte membranes (Hosli et al, 1982b; Hirata et al, 1982) whereas isoproterenol stimulation of β- receptors produces a hyperpolarisation (Hosli et al, 1982b). Further experiments by Hosli et al (1984a; 1984b), again using explant culture and auto-radiography and electrophysiology, suggest that both histamine receptors, (H_1 and H_2) are present on astrocytes and that activation of the two receptor subtypes produces different effects on the astrocyte cell membrane, analogous to the case for adrenergic receptors. Thus, stimulation of H_1 receptors produces a depolarisation, and of H_2 receptors, a hyperpolarisation. Notably in rat cerebral cortex slices H_1 and α_1 receptors are believed to be linked to inositol phospholipid metabolism (Brown et al, 1984; Schoepp et al, 1984) whilst H_2 and β receptors are thought to have effects on the adenylate cyclase system (cf. Brown et al, 1984).

Electrophysiology has also been used to show that some amino acid neurotransmitters, notably glutamate and aspartate, depolarise rat cortical astrocytes in primary culture and that this effect may be mediated by kainate preferring receptors (Kettenman et al, 1984; Bowman and Kimelberg, 1984). However, the evidence is, as yet, equivocal as these experiment were performed in the presence of Na^+ which might have resulted in changes in membrane potential as a result of the Na^+-dependant glutamate transport systems. Also the authors of these two papers (Kettenman et al, 1984; Bowman and Kimelberg, 1984) disagree over the efficacy of GABA to depolarise astrocytes. It is notable that there is no evidence from cyclic nucleotide studies or receptor binding studies that amino-acid

neurotransmitter receptors occur on astrocytes. In particular, Ossola et al (1980) reported that neither [^{3}H]-GABA nor [^{3}H]-muscimol bound to membranes prepared from primary cultures of rat astrocytes.

Highly purified astrocyte cultures (>95%) have been used by Rougon et al (1983) and Evans et al (1984) to demonstrate that a number of peptide hormones and prostaglandin E_1 (PGE_1) stimulate adenylate cyclase activity e.g. vasoactive intestinal peptide (VIP), melanocyte stimulating hormone (MSH) and adrenocorticotropin (ACTH). Other peptides augment the stimulatory effect of noradrenaline on astrocyte cAMP content, e.g. substance P (SP), VIP and somatostatin (SOM) (Rougon et al, (1983) although Evans et al (1984) stated that somatostatin inhibited an isoproterenol induced cAMP increase. In addition morphine and met-enkephalin were shown to antagonise the noradrenaline evoked increase in astrocyte cAMP content (Rougon et al, (1983).

Evidence for the presence of receptors on astrocytes published after Van Calker and Hamprecht (1980) and other than that reviewed above, has been derived from glioma cell lines, bulk separated cells or primary cultures of neonatal chick or rodent brain. These cultures have often been characterised by one or two astrocyte specific immunocytochemical techniques and might therefore contain other cell types. Alternatively, the cultures may have been rigorously characterised and found in fact to contain other cell types (Hansson et al, 1984). However such cultures, and also bovine or equine bulk prepared astrocytes, have been used and suggest that a variety of neurotransmitters stimulate the production of cyclic nucleotides in astrocytes (Table 5.1 e.g.

dopamine, Hansson et al, 1984). Also such cultures have been used to show the binding of various radiolabelled transmitter ligands to astrocytes although notably not to membranes prepared from rat cortical astrocytes (Table 5.1 e.g. [^{3}H]-serotonin, Tardy et al, 1982).

It is important to note that the contamination of primary "astrocyte" cultures has produced misleading data concerning neurotransmitter ligand binding. For example, Ebersolt et al (1981b) suggested that mouse cortical astrocytes in primary culture possess both α_1 and α_2 adrenergic receptors whereas Trimmer et al (1984) have shown that the α_2 receptors found in rat cortical primary astrocyte culture are localised to contaminating meningeal cells. It is also known that the adenylate cyclase activity in meningeal cells in vitro is stimulated by β-adrenergic, purinergic and dopaminergic agonists and also by PGE_1 (Ebersolt et al, 1981b; Evans et al, 1984). Evans et al (1984) also suggested that, as is the case in astrocytes, α-adrenergic receptor agonists could reduce a β-receptor mediated agonist evoked rise in meningeal cell cAMP content. In addition, endothelial cells prepared from bovine cerebral microvessels have muscarinic cholinergic binding sites as detected by [^{3}H]-QNB binding (Estrada et al, 1983). Therefore, when considering the presence of neurotransmitter receptors on astrocytes in primary cultures of less than 100% purity, one must also consider the possibility of, for example, radiolabelled ligands binding to contaminating cells which are probably derived from the meninges. This is also true for any agonist evoked intracellular effects.

This chapter will show for the first time that membranes prepared from neonatal rat cortical astrocytes in vitro express

binding sites for [^{3}H]-DHA, [^{3}H]-serotonin and [^{3}H]-QNB. In addition membranes from meningeal cells in culture possess binding sites for [^{3}H]-DHA and [^{3}H]- serotonin (but not for [^{3}H)-QNB) with similar apparent affinity (Kd values) but lower receptor densities (Bmax) than in astrocyte membranes.

Methods

Cell culture and membrane preparation

Astrocyte-enriched cultures from the neonatal rat cortex were grown to confluence (19-22 d.i.v.) in 60 mm diameter dishes as described in Chapter 4. Meningeal cells were cultivated as before except that for the binding experiments with [^{3}H]-DHA and [^{3}H]-serotonin, the meninges from eight newborn rats were disrupted and plated onto 6 x 250 ml tissue culture flasks (Falcon). The meningeal cell cultures grown in flasks were used at confluence (14 d.i.v.).

Astrocyte and meningeal cell cultures were washed twice with 3 ml of ice-cold PBS per dish or 10 mls of ice-cold PBS per flask and then harvested into ice-cold distilled water. The cells were further disrupted by sonication (Soniprep, MSE Scientific Instruments, amplitude setting 14 for 10 secs.) and allowed to stand on ice for 30 minutes to lyse any vesicles formed and to wash out any endogenous ligand. A crude pellet was prepared for all binding studies by centrifugation at 50,000 g for 30 minutes (Sorvall RC-5B ultracentrifuge, Du Pont Instruments) the pellets being resuspended in assay buffer and aliquots taken for protein

determination (Lowry et al, 1951), using BSA as a standard.

[^{3}H]-DHA binding assay

The binding of [^{3}H]-DHA was determined as in Bylund et al (1978). 100 µg protein aliquots of membrane were incubated with a range (0.02-1nM final concentration) of [^{3}H]-DHA concentrations (Amersham International; specific activity 71 Ci/mmol) in 50 mM Tris-HCl pH 8.0 assay buffer, total volume 1 ml, for 30 minutes at 25°C. The samples were then rapidly filtered under vacuum on Whatman GF/C glass fibre filter discs followed by 2 x 4 ml aliquots of ice-cold assay buffer. Filters were then placed in scintillation vials and dried overnight before the addition of 8 mls of scintillation fluid (Scintran Cocktail T, BDH) and determination of the bound radioactivity in a Beckman scintillation counter (LS 7500, Beckman Instruments) at a counting efficiency of approximately 55%. Specific binding was determined as the difference in [^{3}H]-DHA bound to the filters in the presence or absence of 1 µM l-isoproterenol (Sigma) as a specific displacer. All determinations were made in duplicate or triplicate. Specific binding constituted 26 - 69% of the total binding, mean 50%.

[^{3}H]-serotonin binding assay

[^{3}H]-serotonin binding was performed as in Uzbekov et al (1979). 100 µg protein aliquots of cell membranes were incubated with a range (1 - 100 nM) of [^{3}H]-serotonin concentrations (Amersham International; creatine sulphate salt, specific activity 12.3 Ci/mmol) in 50 mM Tris HCl pH 8.5 assay buffer, total volume 2 mls containing 2 µM pargyline hydrochloride (Sigma), for 10 minutes at

37°C. The assay was terminated by rapid filtering of the membranes over Whatman GF/B filters, followed by 4 x 5 ml washes of ice-cold 50 mM Tris-HCl pH 8.5. The non-specific binding was defined as the binding of [^{3}H]-serotonin in the presence of 20 μM non-radiolabelled serotonin. Specific binding constituted 36 - 70% of the total binding, mean 49%. The radioactivity bound to the filters was determined as for [^{3}H]-DHA binding, the counting efficiency was approximately 63%.

[^{3}H]-QNB binding assay

The method of Gross et al (1981) was used to determine [^{3}H]-QNB binding to 100 μg protein aliquots of cell membranes. The membranes from astrocyte and meningeal cultures were incubated with a range (0.025 - 5 nM) [^{3}H]-QNB (l-isomer, Amersham International, specific activity 40.4 Ci/mmol) for one hour at 25°C in 1 ml 50 mM Na^+/K^+ phosphate buffer (pH 7.4) in the presence or absence of 125 nM atropine sulphate to determine non-specific binding. The samples were then filtered over Whatman GF/B filter discs followed by 3 x 5 ml washes of ice-cold assay buffer. The radioactivity remaining on the filters was counted as before, with an efficiency of approximately 49%. Specific binding constituted 15 - 60% of total binding, mean 38%.

Results

i) β -adrenergic receptors

It is clear from Fig. 5.1 and 5.2 that astrocyte-enriched and meningeal cell cultures possess binding sites for [^{3}H]-DHA, a β-adrenergic receptor ligand. Whilst the results for [^{3}H]-DHA binding to meningeal cell membranes are from a preliminary experiment, both astrocyte and meningeal cell binding sites are seen to possess similar affinities for this ligand (Kd = 88 pM and 56 pM respectively). However, the receptor density (Bmax) for meningeal cell membranes (39 fmol/mg protein) is less than that for astrocyte membranes (51 fmol/mg protein) as determined by Scatchard analysis (Figs 5.1b and 5.2b). The Hill plot of the results for the binding of [^{3}H]-DHA to astrocyte membranes (5.1 c) reveals a slope of less than one (0.72) which suggests that there are multiple binding sites for this ligand on membranes prepared from astrocyte enriched cultures. The lack of data for [^{3}H]-DHA binding to meningeal cell membranes obtained from an experiment designed only to demonstrate the presence or absence of [^{3}H]-DHA binding sites on meningeal cell membranes, restricts its interpretation. However, the Hill plot of this data (Fig. 5.2c) has a slope of close to unity (0.99) which suggests that in contrast to membranes from astrocyte enriched cultures, there is only a single class of [^{3}H]-DHA binding site present on meningeal cell membranes.

ii) Serotonin receptors

[^{3}H]-serotonin binds to both astrocyte and meningeal cell membranes with a similar affinity (Kd = 70 and 76 nM respectively)

A

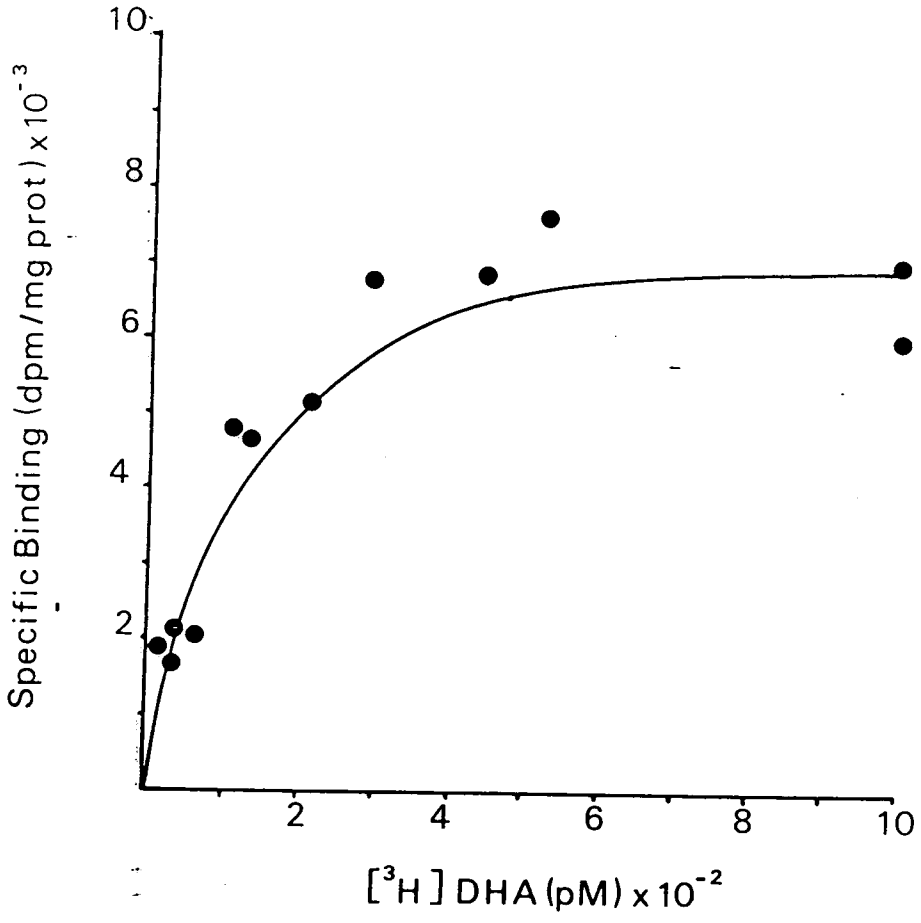
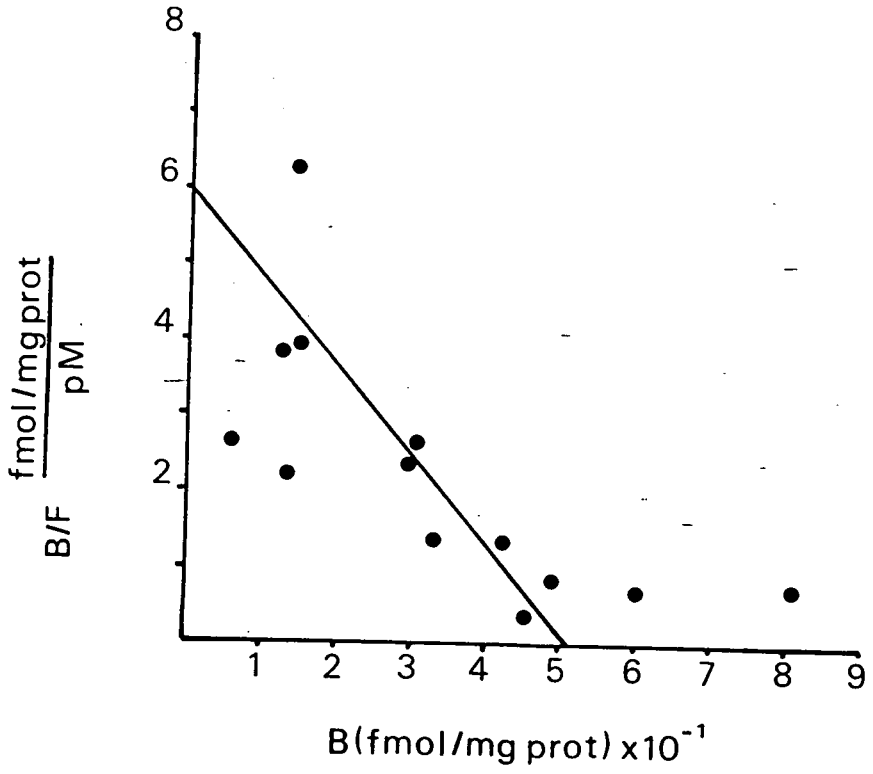


Fig. 5.1

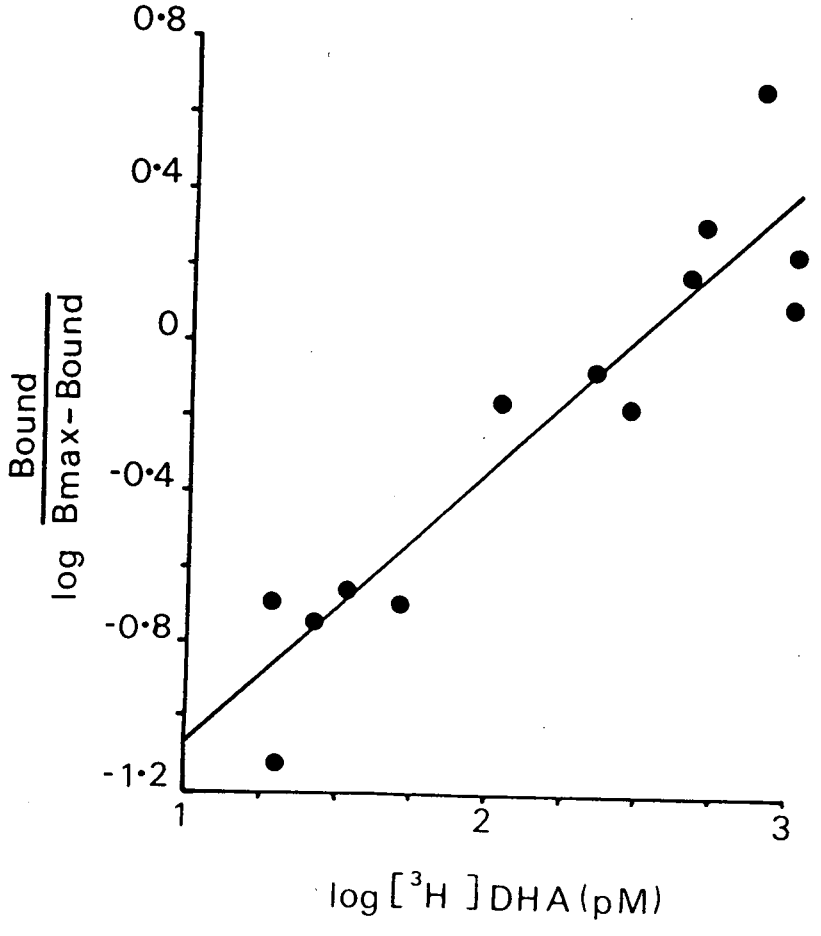
[^{3}H]-DHA binding to astrocyte culture membranes

Four binding experiments were conducted as described in Methods using [^{3}H]-DHA at 2-4 concentrations in duplicate or triplicate in each experiment. The pooled data from all experiments are expressed as a saturation curve (A), a Scatchard plot (B; r=0.74, n=12, p<0.01) and as a Hill plot (C; slope=0.72).

B


8
6
4
2
B/F fmol/mg prot / pM
1
2
3
4
5
6
7
8
9
B(fmol/mg prot) x10⁻¹

C


0·8
0·4
0
-0·4
-0·8
-1·2
log Bound / Bmax-Bound
1
2
3
log [³H] DHA (pM)

A

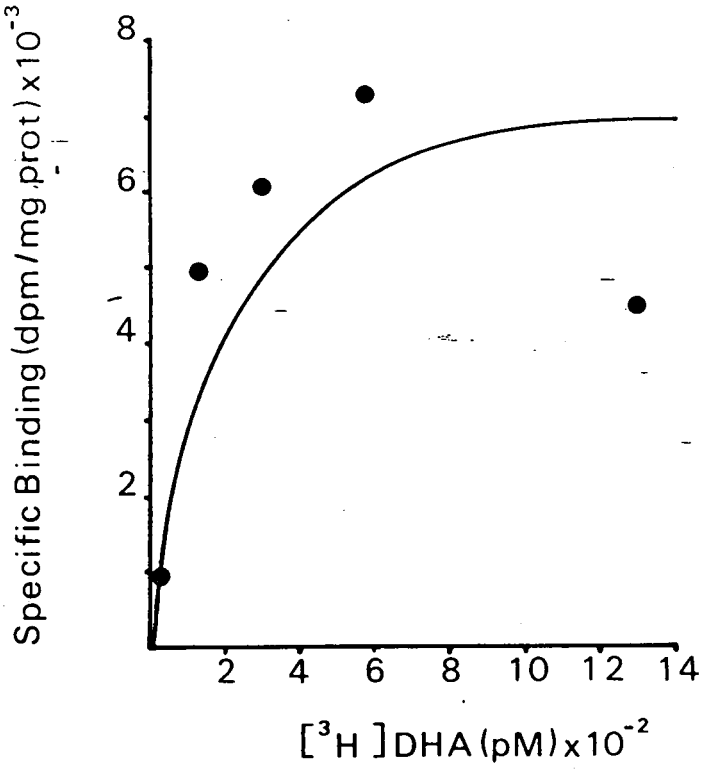
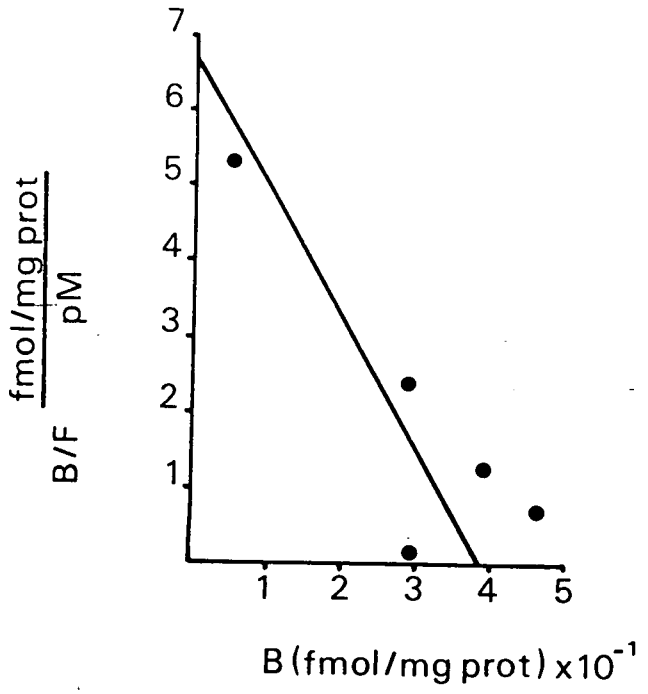


Fig. 5.2

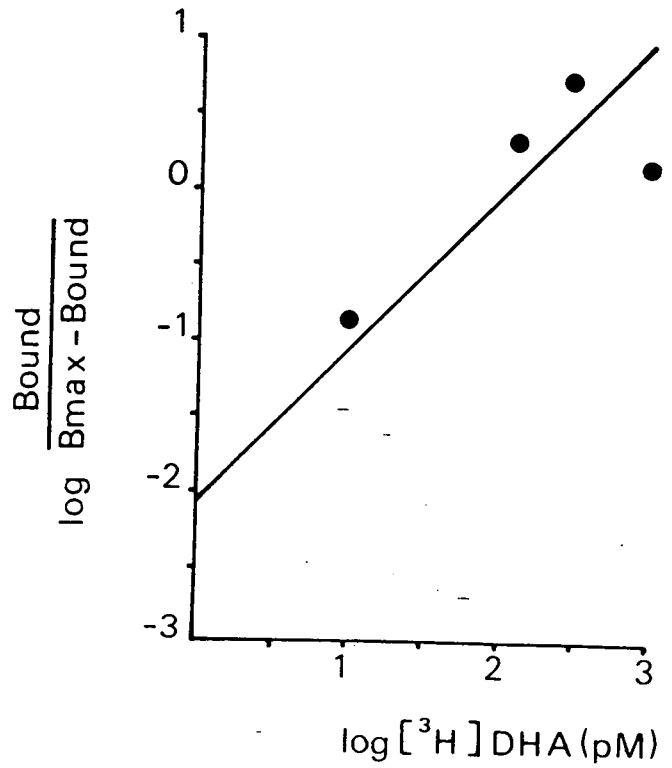
[^{3}H]-DHA binding to meningeal cell culture membranes

Meningeal cell membranes were incubated with 5 concentrations of ligand in duplicate, in one experiment. The results are expressed as a saturation curve (A), a Scatchard plot (B; r=0.83, n=5) and a Hill plot (C; slope=0.99).

B


B/F fmol/mg prot / pM
7
6
5
4
3
2
1
1
2
3
4
5
B (fmol/mg prot) x10⁻¹

C


Bound / log Bmax - Bound
1
0
-1
-2
-3
1
2
3
log [³H] DHA (pM)

A

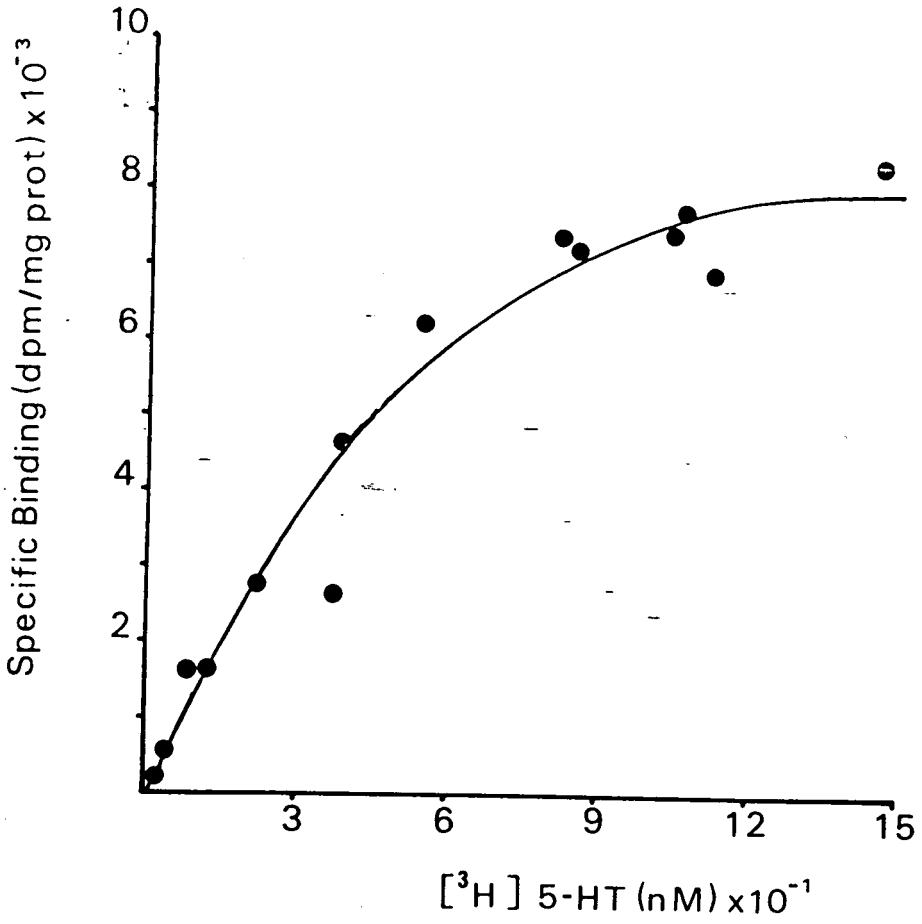
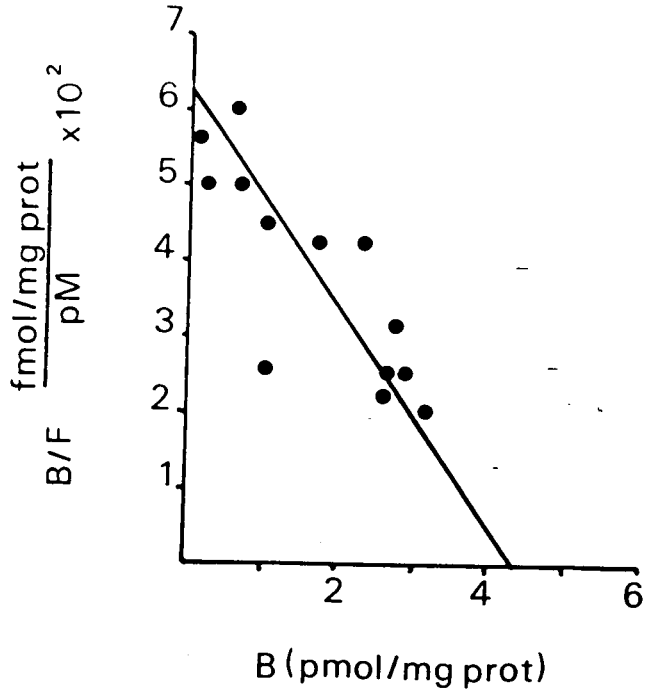


Fig. 5.3

[^{3}H]-serotonin binding to astrocyte culture membranes

This figure shows the pooled results of 4 experiments to determine the binding of [^{3}H]-serotonin to astrocyte membranes using 2-4 concentrations of the ligand in duplicate or triplicate in each experiment. The results are expressed as a saturation curve (A), a Scatchard plot (B; r=0.82, n=14, p=<0.001) and a Hill plot (C; slope=0.98)

B


B/F fmol/mg prot / pM x10²
7
6
5
4
3
2
1
2
4
6
B (pmol/mg prot)

C

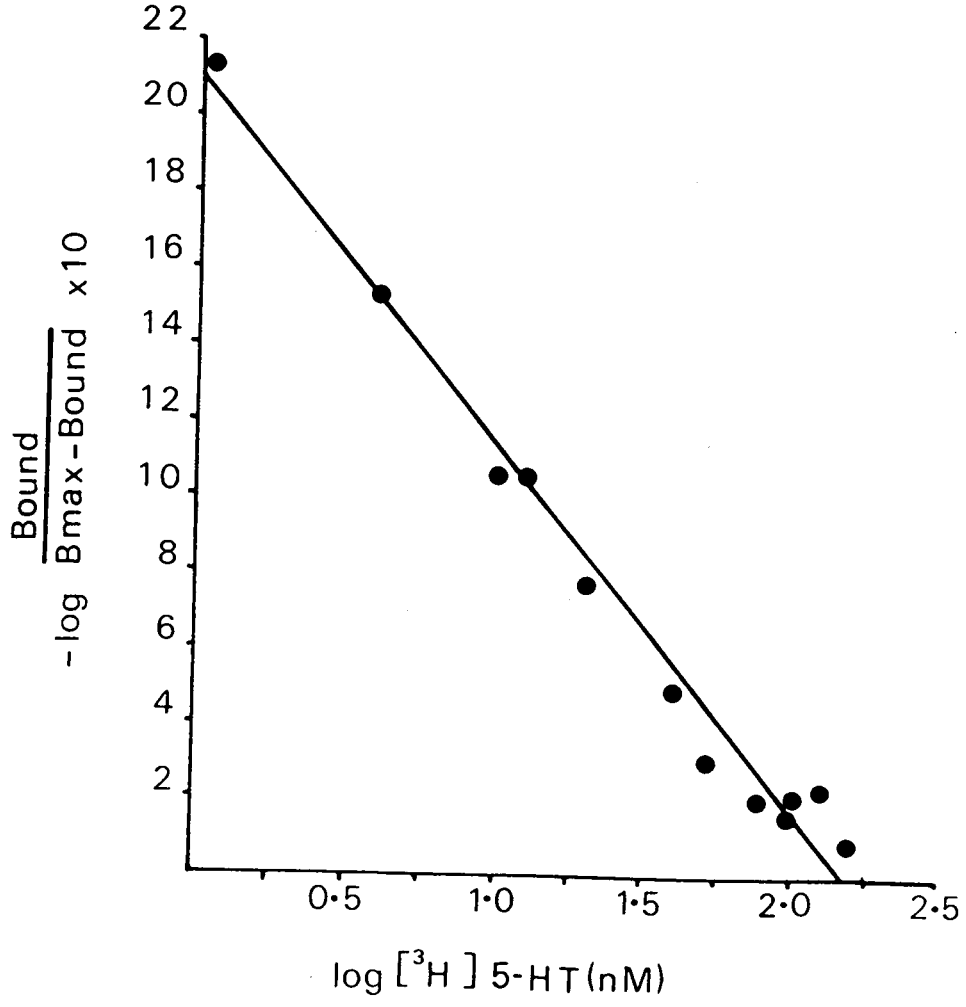
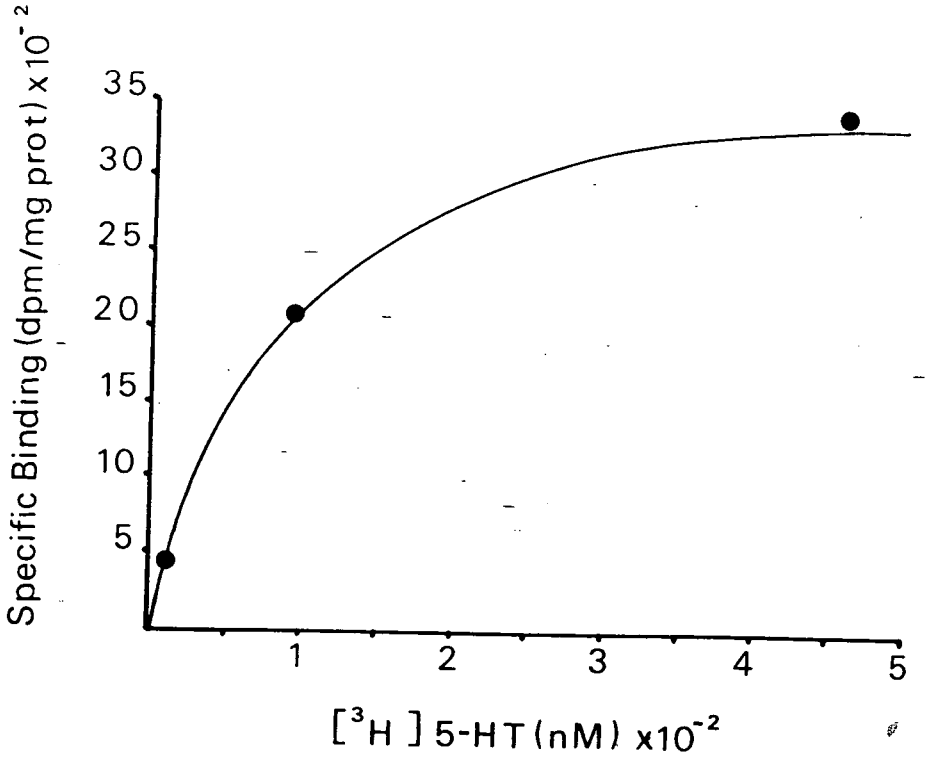

Bound / Bmax-Bound x10
22
20
18
16
14
12
10
8
6
4
2
0·5
1·0
1·5
2·0
2·5
log [³H] 5-HT (nM)

Fig. 5.4

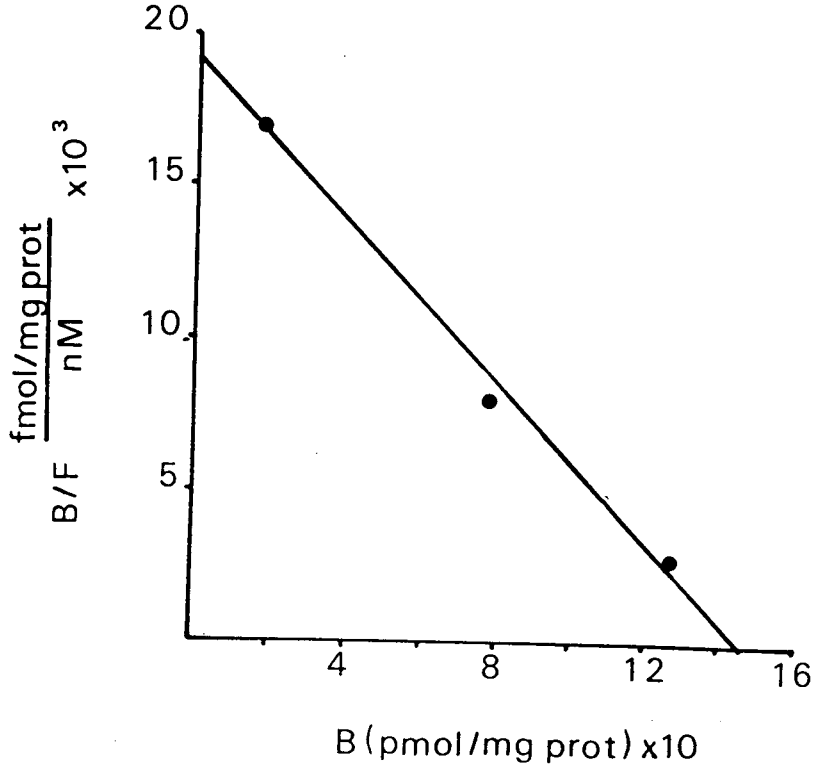
[^{3}H]-serotonin binding to meningeal culture membranes

Meningeal cell culture membranes were incubated with a range of [^{3}H]-serotonin concentration as described in Methods. The figure shows data derived from one experiment performed in duplicate expressed as a saturation curve (A) and a Scatchard plot (B; 4=0.94, n=4, $p<0.01$).

A


Specific Binding (dpm/mg prot) x10⁻²
35
30
25
20
15
10
5
1
2
3
4
5
[³H] 5-HT (nM) x10⁻²

B


B/F fmol/mg prot / nM x10³
20
15
10
5
4
8
12
16
B (pmol/mg prot) x10

A

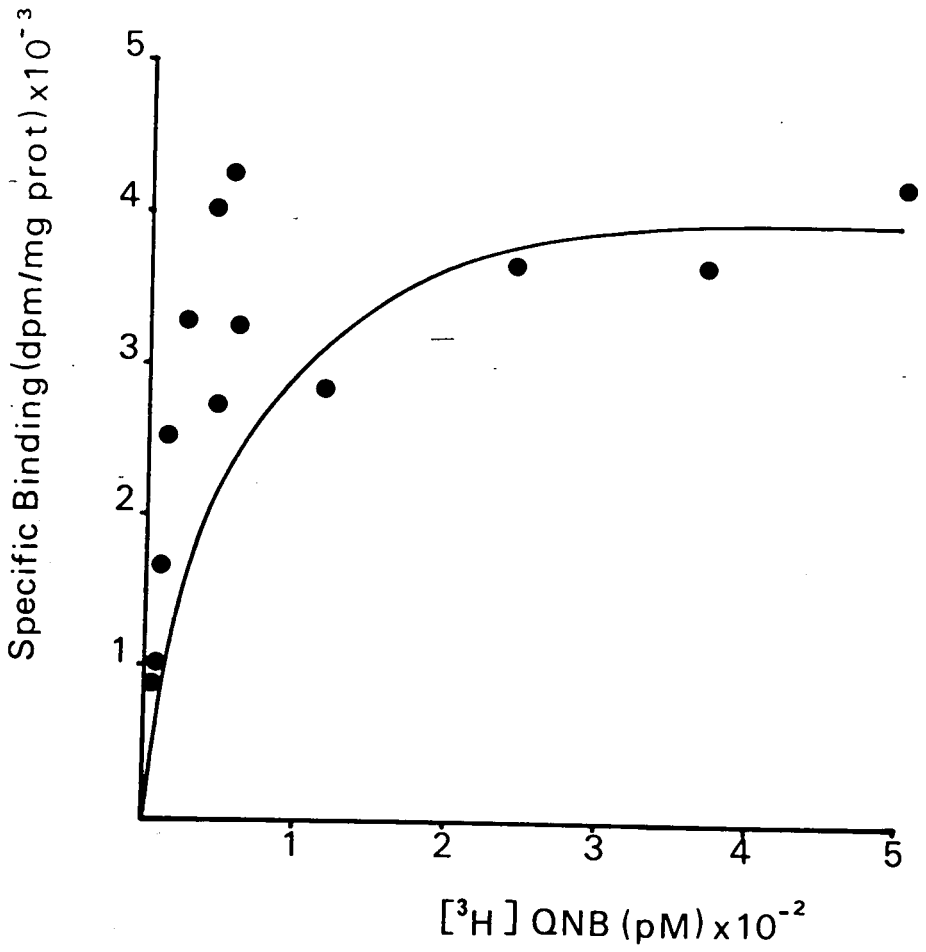
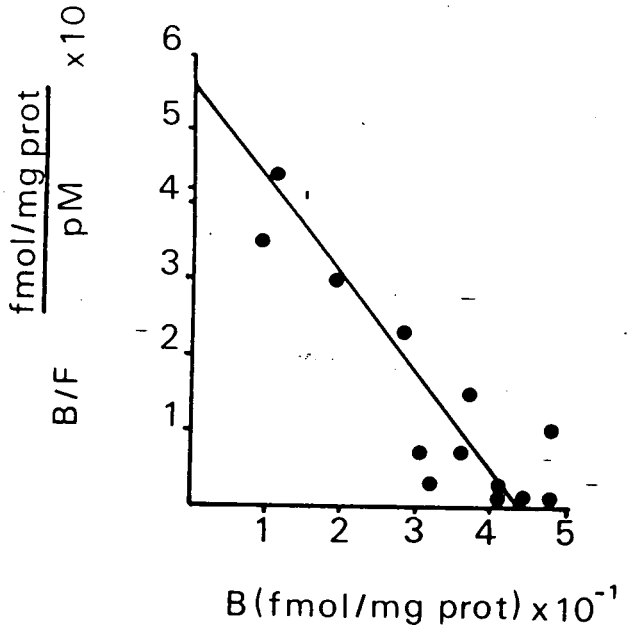


Fig. 5.5

[^{3}H]-QNB binding to astrocyte culture membranes

[^{3}H]-QNB binding to astrocyte membranes was performed as in Methods. The figure represents data pooled from 4 separate experiments each using 2-4 concentrations of ligand in duplicate or triplicate. The results are expressed as saturation curve (A), a Scatchard plot (B; r=0.86, n=13, p<0.01) and a Hill plot (C; slope=1.2).

B


B/F
fmol/mg prot / pM
x10
6
5
4
3
2
1
1
2
3
4
5
B(fmol/mg prot) x10⁻¹

C

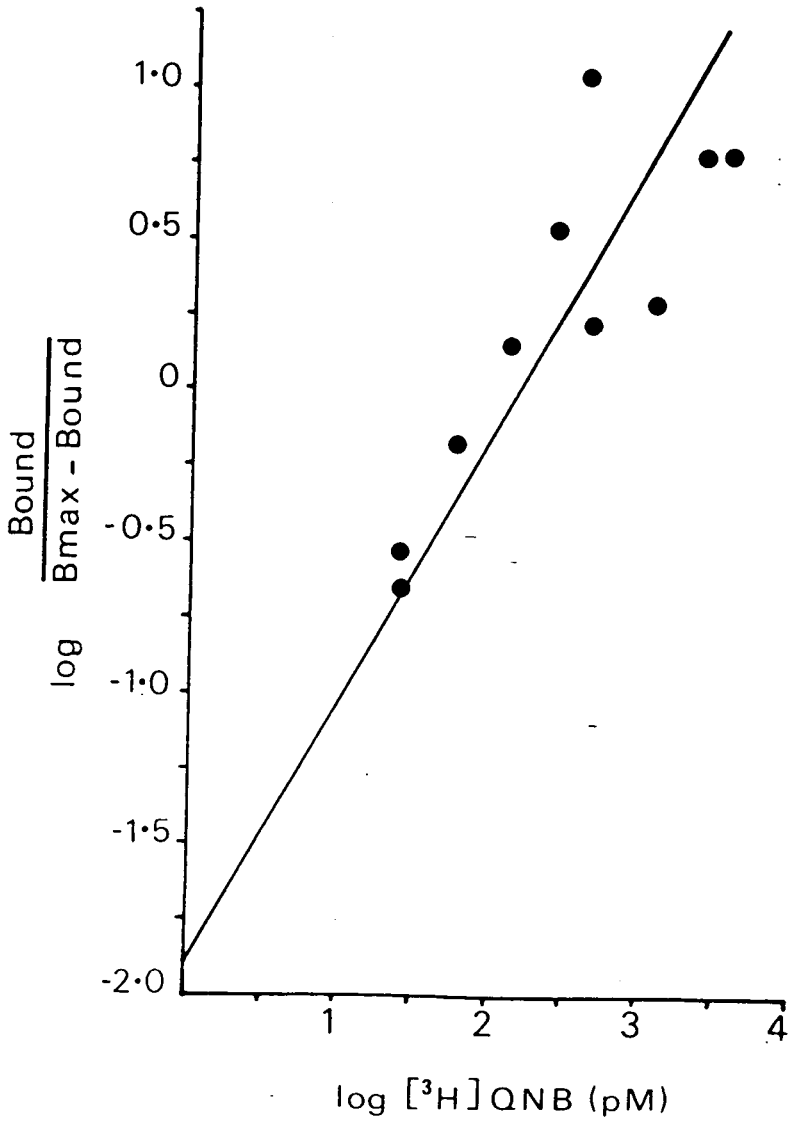

log Bound / Bmax - Bound
1·0
0·5
0
-0·5
-1·0
-1·5
-2·0
1
2
3
4
log [³H]QNB (pM)

Table 5.2 Summary of radioligand binding results

Ligand	Astrocytes		Meningeal Cells	
	Kd (nM)	Bmax (pmol/mg protein)	Kd (nM)	Bmax (pmol/mg protein)
[^{3}H]-DHA	0.088	0.051	0.058	0.039
[^{3}H]-serotonin	70.0	4.4	76.0	1.5
[^{3}H]-QNB	0.079	0.044	-	-

Legend

The kinetic parameters shown here were determined by Scatchard analysis of data presented elsewhere. - = no specific binding of this ligand

but with markedly lower receptor density on meningeal cells (Bmax = 1.5 pmol/mg protein compared to 4.4 pmol/mg protein as derived from Scatchard analysis (Figs 5.3b and 5.4b). [^{3}H]-serotonin binds to a single population of binding site on astrocytes as indicated by the Hill analysis of this data (Fig. 5.3c) revealing a slope close to unity (0.98).

iii) Muscarinic receptors

When [^{3}H]-QNB binding to cell culture membranes is considered (Fig. 5.5) it is evident that in this case only astrocytes possess binding sites for this muscarinic cholinergic ligand. No specific binding of [^{3}H]-QNB to meningeal cell membranes was seen over the range 1.3 - 327 pM [^{3}H]-QNB in two separate experiments. Hill analysis of the data (Fig. 5.5c) suggests that [^{3}H]-QNB binds to a single site on astrocyte membranes as the slope (1.2) is not markedly different from one. The affinity of this site for [^{3}H]-QNB is Kd=79 pM and receptor density (Bmax) = 44 fmol/mg protein.

The results of the experiments described here are summarised in Table 5.2.

Discussion

The principal aim of this series of studies was to determine whether rat cortical astrocytes in primary culture possess binding sites for neurotransmitters. The results presented here show that membranes prepared from cortical astrocytes possess binding sites for three neurotransmitter ligands; [^{3}H]-DHA, [^{3}H]-serotonin and [^{3}H]-QNB. This suggests the presence of β-adrenergic, serotonergic and muscarinic cholinergic receptors on these cells. In addition, membranes prepared

from meningeal cells in vitro express binding sites for two of these ligands, [^{3}H]-DHA and [^{3}H]-serotonin and approximate Kd and Bmax calculated. Thus experiments to determine the functional role of these receptors in astrocyte enriched cultures, which are know to contain meningeal cells (Chapter 3) must also consider possible actions of neurotransmitter agonists on meningeal cells.

The binding of [^{3}H]-DHA to membranes prepared from neonatal mouse cortical cultures has been described by Ebersolt et al (1981b) and to intact chick astrocytes in vitro by Maderspach and Fajszi (1983). However, to the author's knowledge, the data presented here constitute the first report of the binding of this ligand to rat cortical astrocyte cultures.

There is some variation in the previously reported Kd values for [^{3}H]-DHA binding to astrocytes. Ebersolt et al (1981b) state that [^{3}H]-DHA binds to mouse astrocyte membranes at a single site with a Kd of 500 pM and Bmax of 145 fmol/mg protein. In chick astrocyte cultures the binding of [^{3}H]-DHA is also to a single site but with an affinity of 44 pM and Bmax of 3.3 fmol/10^6 cells (approximately 33 fmol/mg protein; Maderspach and Fajszi, 1983). The value for Kd presented here (88 pM) is thus within the range of those previously reported for [^{3}H]-DHA binding to astrocytes. It is also very similar to those reported for the binding of [^{125}I]-pindolol derivatives (β-adrenergic antagonists) to rat cortical astrocytes in vitro i.e. 4.4 - 52 pM (Harden and McCarthy, 1982; McCarthy, 1983; Trimmer et al, 1984) and to C6 glioma, i.e. 5.6 - 150 pM (Schmitt and Pochet, 1977; Barovsky and Brooker, 1980; Shitara et al, 1984).

However, not all of the [^{3}H]-DHA binding to "astrocyte" membranes can be attributed to astrocytes alone as preliminary data

suggests that [^{3}H]-DHA also binds to meningeal cell membranes. In this respect it is notable that a Hill analysis of the [^{3}H]-DHA binding to astrocyte membranes suggests that there are more than one class of [^{3}H]-DHA binding sites present on these membranes, with differing affinities for the ligand. When it is considered that Trimmer et al (1984) showed that astrocytes in vitro express only the β_1-adrenergic receptor subtype (the β_2-receptor subtype being expressed by meningeal cells) and that [^{3}H]-DHA has different affinities for the two B-receptor subtypes (U'Prichard et al, 1980), it is probable that some of the [^{3}H]-DHA binding to "astrocyte" membranes is to β_2-receptros on meningeal cell membranes. Membranes prepared from both astrocyte-enriched and meningeal cell cultures also possess binding sites for [^{3}H]-serotonin. In the case of astrocyte membranes, the binding is to a single site (K_d = 70nM) as shown by Hill analysis

Previous evidence for the binding of [^{3}H]-serotonin to astrocytes or meningeal cells in vitro is very limited. Indeed for meningeal cells there is none. Hertz et al (1979) showed that [^{3}H]-serotonin binds to intact mouse primary astrocyte cultures, although saturation binding kinetics were not determined. In the report of Hertz et al (1979) both 11 nM and 33 nM [^{3}H]-serotonin were seen to bind to the astrocytes with a value for specific binding of 50 fmol/mg protein. From this evidence the authors suggest that the Kd value for the [^{3}H]-serotonin binding site was probably below 11 nM. A later study by Tardy et al (1982) also examined the binding of [^{3}H]-serotonin to mouse astrocyte cultures and derived a value for the affinity of the binding site of Kd = 15 nM and receptor density Bmax = 180 fmol/mg protein. A similar value of affinity (10 nM) for [^{3}H]-serotonin binding to astrocytes has been given by Fillion et al (1983) who examined the binding of this ligand to astrocytes bulk prepared from the horse striatum.

Binding of the muscarinic cholinergic receptor ligand [^{3}H]-QNB to membranes prepared from astrocytes in culture indicate that there is a single class of binding site for [^{3}H]-QNB on astrocytes with an affinity of 79 pM and Bmax of 44 fmol/mg protein. Again, this is the first report of such a binding site to membranes prepared from rat cortical astrocytes in primary culture and other evidence for the expression of such a site on astrocytes is very limited. Repke and Maderspach (1982) show that [^{3}H]-QNB binds to intact astrocyte cultures prepared from chick hemispheres (i.e. an intact cell preparation) with a Kd of 94 pM and Bmax of 76 fmol/mg protein. [^{3}H]-QNB also binds to 1321N1 astrocytoma cell membranes with a Kd in this case of 6.3 pM (Evans

et al, 1984). The kinetic parameters given here (Table 5.2) are thus within the range of previously published results.

In contrast, there is no specific binding of [^{3}H]-QNB to meningeal cell membranes. However, Estrada et al (1983) and Grammas et al (1983) have reported that [^{3}H]-QNB binds to endothelial cells from bovine cerebral cortex and to microvessels from the rat cerebral cortex respectively. It is possible that the lack of [^{3}H]-QNB binding to meningeal cell membranes may reflect a paucity of endothelial cells in meningeal cell cultures, as previously discussed (Chapter 3).

Whilst the binding of these three neurotransmitter ligands to astrocytes in vitro has not been extensively studied prior to this report, their existence had been inferred (but not proved) by studies of the effects of neurotransmitter agonists on cyclic nucleotide levels (see Introduction). In fact the stimulation of cyclic nucleotide second messengers by transmitter agonists constitutes the major source of evidence that neurotransmitter binding sites on astrocytes represent functional receptors. By far the most studied receptor from this respect has been the β-adrenergic receptor. It is now well accepted that β-adrenergic receptor agonists stimulate cAMP production in a variety of astrocyte systems (see Introduction). Indeed isoproterenol (a β-receptor agonist) stimulates the production of cAMP (70 fold) in similarly prepared astrocytes to those used in this study (Murphy et al - submitted for publication). This latter observation suggests that the β-adrenergic binding site found on astrocyte membranes may be a functional receptor although, as meningeal cells also respond (11 fold) to isoproterenol by cAMP production (Murphy et al - submitted),

an additional stimulation of these cells in astrocyte cultures cannot be excluded.

The case for the [^{3}H]-serotonin and [^{3}H]-QNB binding sites found on astrocyte membranes constituting receptors linked to an intracellular second messenger system modulating a physiological response, is not as well established. Both primary cultures of mouse cortical astrocytes (7-14 d.i.v.) and astrocytes separated from the horse striatum, have been reported to show increases in the level of cAMP after stimulation by serotonin (Fillion et al, 1980; 1983). However, it is notable that the action of serotonin on cAMP content of astrocytes is equivocal. Ebersolt et al (1981c) showed no stimulation of adenylate cyclase activity by serotonin in older mouse cortical cultures (18 - 20 d.i.v.). Also, Bottenstein and de Vellis (1978) reported that serotonin affected the cAMP levels of C6 glioma cells only at high levels (300 μM) and that the effect observed was a decrease in cAMP. More recently Sugino et al (1984) have shown that serotonin causes an increase in the intracellular Ca^{2+} concentration in a subclone of C6 gliomoa cells. Furthermore, in this laboratory it has been shown that serotonin elicits an increase in inositol phospholipid turnover in primary cultures of rat cortical astrocytes (Pearce et al, 1985c). It appears therefore that the [^{3}H]-serotonin binding site on astrocyte membranes reported in this chapter may be linked to a second messenger system which might effect physiological responses in astrocytes (Chapter 6).

As was the case for serotinergic sites on astrocytes, stimulation of the muscarinic cholinergic receptor on these cells has been reported to affect the activity of adenylate cyclase

although in this case, the effects are inhibitory. Thus activation of the muscarinic cholinergic receptor on C6 cells by agonists such as carbachol reduces the basal level of cAMP and also cGMP (Bottenstein and de Vellis, 1978). It has also been reported that carbachol reduces the noradrenaline or isoproterenol-evoked rise in cAMP in glioma cell lines by a Ca^{2+} dependent process (Gross and Clark, 1977; Bottenstein and de Vellis, 1978; Evans et al, 1984). In 1321N1 astrocytoma cells, carbachol is thought to reduce cAMP levels by activation of phosphodiesterase(s) (cf Evans et al, 1984). Recent work in our laboratory has shown that carbachol also reduces the isoproterenol evoked cAMP production in astrocyte primary cultures (Murphy et al - submitted) in this case directly via an inhibitor of adenylate cyclase. Furthermore, it has also been shown in our laboratory that carbachol causes a Ca^{2+} dependent, atropine sensitive increase in the accumulation of [^{3}H] inositol phosphates in astrocyte cultures pre-labelled with [^{3}H] myo-inositol (Pearce et al, 1985c). These data indicate that astrocytes possess both M1 and M2 muscarinic receptor subtypes which are linked to either inositol phospholipid metabolism or adenylate cyclase respectively (Evans et al, 1984). In contrast, as predicted by the lack of [^{3}H]-QNB binding to meningeal cell membranes, carbachol has only a slight stimulatory effect on inositol phospholipid metabolism in meningeal cells (Pearce et al, 1985c) and does not reverse isoproterenol-stimulated cAMP levels (Murphy et al - submitted).

Work performed in our own and other laboratories, therefore suggests that the binding sites shown to be present on astrocytes in vitro (this chapter) may be linked to second messenger systems. However, the possible intracellular effects of activation of these receptors subsequent to second messenger production are in the main unknown. Most of the known effects of receptor activation

have been reviewed by Van Calker and Hamprecht (1980). Briefly, in C6 glioma cells, β-adrenergic stimulation increases the activity of lactate dehydrogenase, ornithine decarboxylase, CNPase and GPDH and an increase in carbonic anhydrase activity in astrocyte primary cultures. The significance of these changes is, as yet, unknown. β-adrenergic agonists also induce "morphological differentiation" of astrocytes in vitro, i.e. the cells change from a flattened polygonal shape to a bipolar or astrocyte-like morphology with extensive processes. Of particular interest have been the reports that some neurotransmitters or their agonists can stimulate glycogenolysis in astrocytes (see Chapter 6). The glycogen stores of astrocytes have already been suggested to constitute a pool of metabolic intermediates which can be used by neurons. Moreover, the metabolism of astrocyte glycogen can be influenced by neuronal activity (Chapters 1 and 4). The next chapter will therefore examine the intracellular consequence of astrocyte neurotransmitter receptor activation reflected by changes in the metabolism of the glycogen stores of astrocyte and meningeal cell cultures.

CHAPTER 6

The effects of neurotransmitter agonists and antagonists on astrocyte glycogen stores

Introduction

In Chapter 1 the concept of neuron/glia signalling was introduced. It was shown in Chapter 4 that astrocytes in primary culture do respond to one such putative signal i.e. a change in $[K^+]_o$, by alterations in glycogen metabolism. Astrocyte glycogen stores have been suggested to constitute an important reserve pool of metabolic intermediates for neurons, indeed, in the CNS, glycogen is localised predominantly to astrocytes (see Chapters 1 and 4). It was also suggested that astrocytes might respond to other signals released from neurons such as neurotransmitters, by alterations in glycogen metabolism. Chapter 5 showed that astrocyte cultures possess binding sites for at least three neurotransmitters, noradrenaline (β-adrenergic), acetycholine (muscarinic) and serotonin. However, as previously stated, the presence of receptors for neurotransmitters on cell membranes implies nothing about what, if any, intracellular consequences might occur after the receptors are activated. This chapter will examine the effects of activation of these particular receptors on the glycogen metabolism of neonatal rat cortical astrocytes in primary culture.

Previous studies, albeit few, have suggested that the glycogen stores of astrocytes in vitro can be influenced by neurotransmitters. The most studied transmitter in this respect is noradrenaline and there are a few reports of serotonin, but not of acetylcholine effects on astrocyte glycogen stores. For example, stimulation of adrenergic receptors has been shown to evoke glycogenolysis in C6 cells (Opler and Makman, 1972; Browning et al, 1974; Passoneau and Crites, 1976).

In addition, noradrenaline, as well as histamine, serotonin and adenosine, stimulate the hydrolysis of [^{3}H]-glycogen newly formed from [^{3}H]-glucose in rat hemisphere primary cultures (Magistretti et al, 1983).

Frequently, the control of astrocyte glycogen metabolism by neurotransmitters is considered to be via a cAMP dependant mechanism. For example, noradrenaline and isoproterenol are known to increase cAMP in astrocytes via β-adrenergic receptors (see Van Calker and Hamprecht, 1980). Passoneau and Crites (1976) observed that the noradrenaline evoked increase in cAMP accumulation in C6 cells was mirrored by an increase in GPa activity, a reduction in GSa activity and a consequent reduction in total glycogen content. Also Cummins et al (1983b) showed that the noradrenaline evoked glycogenolysis in (Herpes virus transformed-) astrocyte cultures was blocked by the adrenergic receptor antagonist propranolol. Furthermore, the β-receptor agonist isoproterenol causes glycogenolysis in these cells whilst the α-adrenergic receptor agonist phenylephrine was ineffective. However, a direct link between cAMP and glycogenolysis in astrocytes has not been shown. Moreover, transmitter control of astrocyte glycogen is not always regarded as being cAMP dependant. Magistretti et al (1983) proposed that histamine evoked glycogenolysis in rat astrocyte cultures through a cAMP independant manner. In addition cAMP is not thought to be involved in the control of glycogen metabolism in C6 cells by glucose (Passoneau and Crites, 1976) or in the K^+ evoked glycogenolysis seen in mouse cortical slices (Ververken et al, 1982)

Notably in the liver it has recently become clear that adrenergic control of glycogen stores involves non-cAMP linked,

α-receptor mediated effects as well as cAMP linked, β-receptor effects (Williamson et al, 1981). Indeed in some species such as the rat, non-cAMP linked, α-adrenergic receptor control of hepatic glycogen metabolism prodominates over B-adrenergic control (Kunos, 1984). Moreover, when considering the neurotransmitter control of the glycogen stores of astrocytes in vitro it is important to note that Kunos (1984) suggests that an increased contribution of β-adrenergic receptor mediated control of glycogenolysis over α-adrenergic receptor control may result from a lack of cellular differentiation. This is of particular interest as the majority of studies regarding neurotransmitter control of astrocyte glycogen stores have used C6 cells or virus-transformed astrocytes which might be regarded as relatively undifferentiated or dedifferentiated cells.

Therefore, while increases in intracellular levels of cAMP have been reported to stimulate glycogenolysis in astrocytes, there is no direct evidence to support the idea that neurotransmitters influence astrocyte glycogen metabolism through such a mechanism.

The experiments described in this chapter were designed to answer three basic questions.

a) Does activation of any of the three neurotransmitter receptors known to be present on the membranes of rat cortical astrocytes in vitro (Chapter 5) alter the metabolism of glycogen?

b) Are any effects on astrocyte glycogen stores specific to a particular receptor or do they represent a general consequence of receptor activation?

c) Is the noradrenergic control of glycogen metabolism in astrocytes in vitro mediated by α- or β-adrenergic receptors?

As astrocyte-enriched cultures are known to be contaminated with cells presumably derived from the meninges (Chapter 3), and it is known that meningeal cells in vitro contain glycogen (Chapter 4) and express neurotransmitter receptors (Chapter 5), the effects of transmitter receptor activation on the glycogen stores of meningeal cells were also examined.

This chapter will show that:-

a) Enhanced glycogenolysis in astrocyte cultures is not a general consequence of receptor activation but is confined to two of the three receptor types examined, i.e. adrenergic and serotonergic but not muscarinic cholinergic.

b) In the case of the noradrenergic control of astrocyte glycogen stores, the action of noradrenaline cannot be sufficiently explained by mediation through either α or β-receptors alone but it is clear that β-receptor stimulation is not the predominant route of noreadrenaline evoked glycogenolysis.

In addition:

a) Meningeal cells in vitro respond to noradrenaline by a reduction in glycogen stores but in this case both α and β-adrenergic agonists are capable of eliciting glycogenolysis.

b) Cells in astrocyte-enriched and meningeal cultures may release a monoamine transmitter (possibly serotonin in astrocyte cultures) which exerts an endogenous control of glycogen stores.

Methods

For these experiments, confluent astrocyte-enriched and meningeal cell cultures were prepared in 60 mm diameter dishes (Falcon) as described in Chapter 3. The cell cultures were pre-incubated for 2 hours in medium supplemented with 20 mM glucose to maximise glycogen content as described in Chapter 4. However, in the case of reserpine treatment of cultures, the cells were incubated overnight (16 - 17 hours) with 10 μM reserpine in buffer containing 20 mM glucose (see Chapter 4) and were not pre-incubated in glucose-supplemented medium.

After pre-incubation with glucose-supplemented medium or 10 μM reserpine in buffer, the cultures were washed with 3 mls of buffer and incubated (37° in 35.5% CO_2/air) for one hour in 5 mls of fresh buffer containing a variety of pharmacological agents. Where the effects of monoamine neurotransmitters, their agonists or antagonists were being examined, pargyline hydrochloride (100 μM), tropolone (100 μM) and ascorbic acid (1 mM) were added to both the wash and experimental buffer to inhibit the breakdown of amines by monoamine oxidase, catechol-o-methyl transferase and oxidation respectively. In all experiments control cultures were incubated in buffer alone ± metabolic inhibitors as appropriate. Where the effect of an antagonist on the agonist-evoked change in glycogen level of the cell cultures was being examined, the effects of the agonist and antagonist alone were also tested during the same experiment. Thus all statistical comparisons reported here were performed using a matched pair, two-tailed t-test as in Chapter 4 (Snedecor and Cochran, 1967).

All chemicals were obtained from the Sigma Chemical Company except phentolamine (CIBA Geigy), prazosin (Pfizer Ltd) and forskolin (Hoechst).

Results

The effects of neurotransmitter agonists on the glycogen content of cell cultures

Fig. 6.1 shows the effects of neurotransmitter agonists on the glycogen content of astrocyte-enriched and meningeal cell cultures. Only noradrenaline (50 μM) is seen to evoke a statistically signficant change in the glycogen content of astrocyte cultures when compared to control (a 33% decrease), whereas serotonin and carbachol are without effect. Noradrenaline also causes a statistically significant net glycogenolysis (20%) in meningeal cell cultures although the effect is less than that in astrocyte cultures. The muscarinic cholinergic agonist carbachol evokes apparent small decreases in astrocyte glycogen stores at each of four different concentrations, 500 μM (16%), 5 μM (6%), 1 μM (9%) and 0.5 μM (18%) but none of these are statistically significant when compared to controls run in parallel. Similarly, 50 μM carbachol causes an apparent but not statistically significant change in meningeal cell glycogen (a 4% increase). The apparent increase in astrocyte glycogen content seen after incubation with 50 μM serotonin is not statistically significant when compared to control.

When astrocyte and meningeal cell cultures are exposed to specific adrenergic receptor subtype agonists (Fig. 6.2a) it is evident that neither the glycogenolysis evoked by 50 μM isoproterenol (β-receptor agonist, 5%) nor by 50 μM phenylephrine (α-receptor agonist, 13%) in astrocyte cultures are statistically significantly less than control values. However, if meningeal cell cultures are exposed to 50 μM isoproterenol there is a statistically significant

Fig. 6.1

Effects of neurotransmitter agonists on cell culture glycogen content I

Astrocyte-enriched and meningeal cell cultures were incubated in buffer containing 50 µM noradrenaline (NA), 50 µM serotonin (5-HT) or 500 µM carbachol (CAR) and the glycogen content determined as in Methods. n is not less that 4 for all determinations where n represents the number of treated cultures. The data were obtained from the number of experiments shown.

* represent a value statistically significantly different from control ($p<0.05$ or less)

Error bars indicate the standard error of the mean.

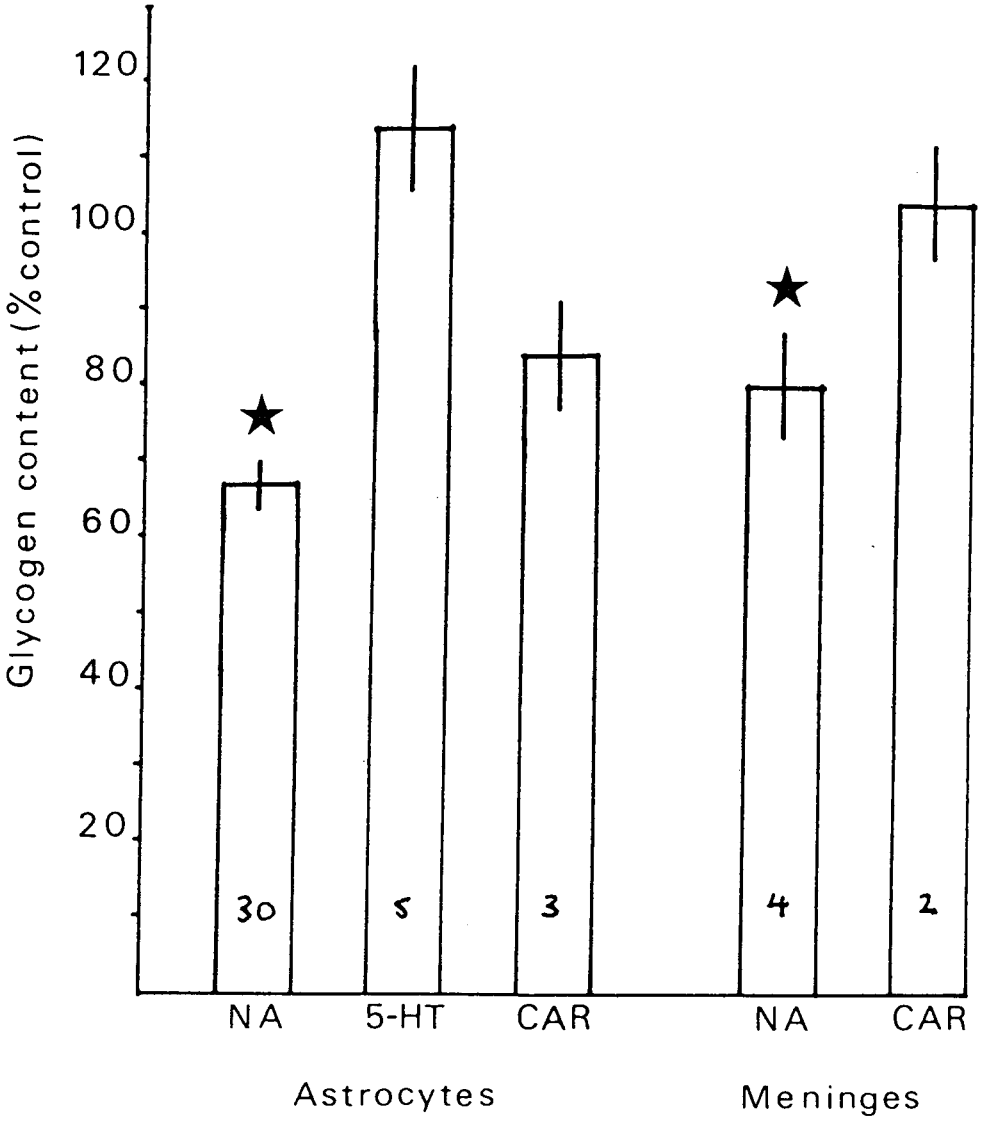

Glycogen content (% control)
120
100
80
60
40
20
30
5
3
4
2
NA
5-HT
CAR
NA
CAR
Astrocytes
Meninges

Fig 6.2a

Effects of neurotransmitter agonists on cell culture glycogen content II

Astrocyte-enriched and meningeal cell cultures were incubated in buffer containing 50 μM noradrenaline (NA), 50 μM isoproterenol (ISO), 50 μM Phenylephrine (PHE), 1 mM dibutyryl cAMP (DB) or 20 μM forskolin (FOR) and glycogen content determined as in Methods. n is not less than 11 for all determinations, the data being derived from the number of experiments shown.

* represents a value statistically significantly different from control ($p<0.05$ or less)

Error bars indicate the standard error of the mean

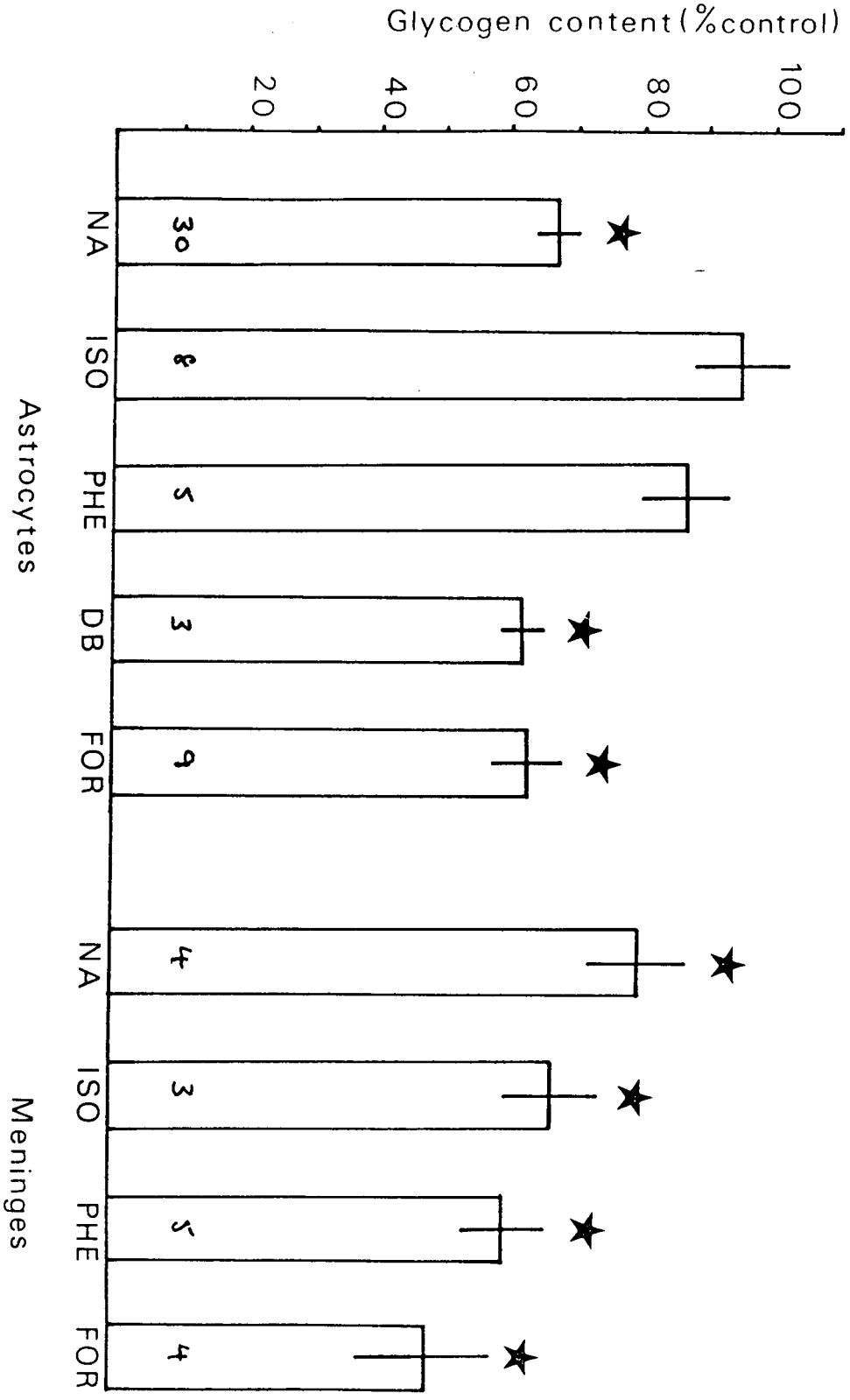

Glycogen content (%control)
20
40
60
80
100
NA
ISO
PHE
DB
FOR
NA
ISO
PHE
FOR
30
8
5
3
9
4
3
5
4
Astrocytes
Meninges

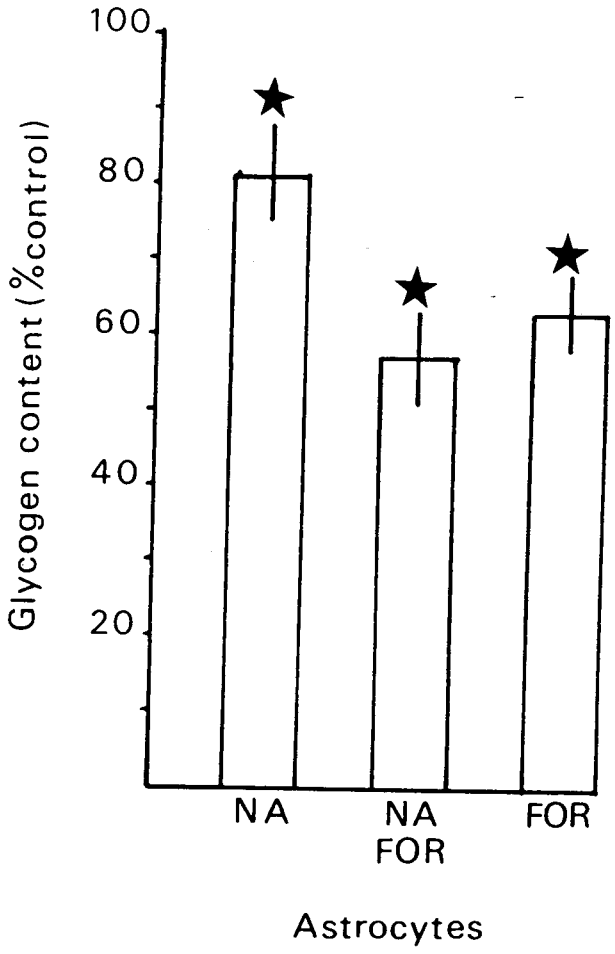
Fig. 6.2b

Effect of noradrenaline in the pres ence or absence of forskolin on astrocyte glycogen content

Astrocyte-enriched . . . . cultures were incubated in 50 μM noradrenaline (NA), 50 μM noradrenaline and 20 μM forskolin (FOR), and 20 μM forskolin alone. Glycogen content was measured as described in Methods. n for this series of experiments is 10 in each case, the data being derived from 5 experiments.

* represents a value statistically significantly different from control ($p<0.05$ or less)

Error bars indicate the standard error of the mean


100
80
60
40
20
Glycogen content (%control)
NA
NA FOR
FOR
Astrocytes

decrease (33%) in glycogen content. There is also a decrease (40%) in glycogen stored in meningeal cell cultures after incubation with 50 μM phenylephrine.

Compounds which raise the level of intracellular cAMP without interaction with transmitter receptors cause marked reductions in the glycogen content of astrocyte and meningeal cell cultures (Fig 6.2a). 1mM dibutyryl cAMP (dBcAMP) causes a 38% loss of astrocyte glycogen and 20 μM forskolin evokes a 37% decrease. The effect of forskolin is even greater on the glycogen stores of meningeal cells (52%).

Also when 20 μM forskolin is added to astrocyte cultures in combination with 50 μM noradrenaline (Fig 6.2b) the resulting glycogen breakdown (57% of control) is not significantly greater than that seen in the presence of 20 μM forskolin alone in this series of experiments (63% of control). This may indicate that forskolin and noradrenaline do not evoke glycogenolysis by completely independent means or, alternatively, that the breakdown evoked by 20 μM forskolin represents maximal glycogenolysis.

The effects of receptor antagonists on adrenergic agonist-evoked changes in the glycogen content of cell cultures

Fig. 6.3. shows the results of experiments where the α and β-adrenergic receptor antagonists phentolamine and propranolol were used in attempts to block the noradrenaline evoked glycogenolysis seen in astrocyte and meningeal cell cultures. In astrocytes, 200 μM propranolol did not reverse the effect of noradrenaline on glycogen stores but it is clear that propranolol itself causes a breakdown of glycogen. Phentolamine (200 μM) also produces a statistically significant reduction

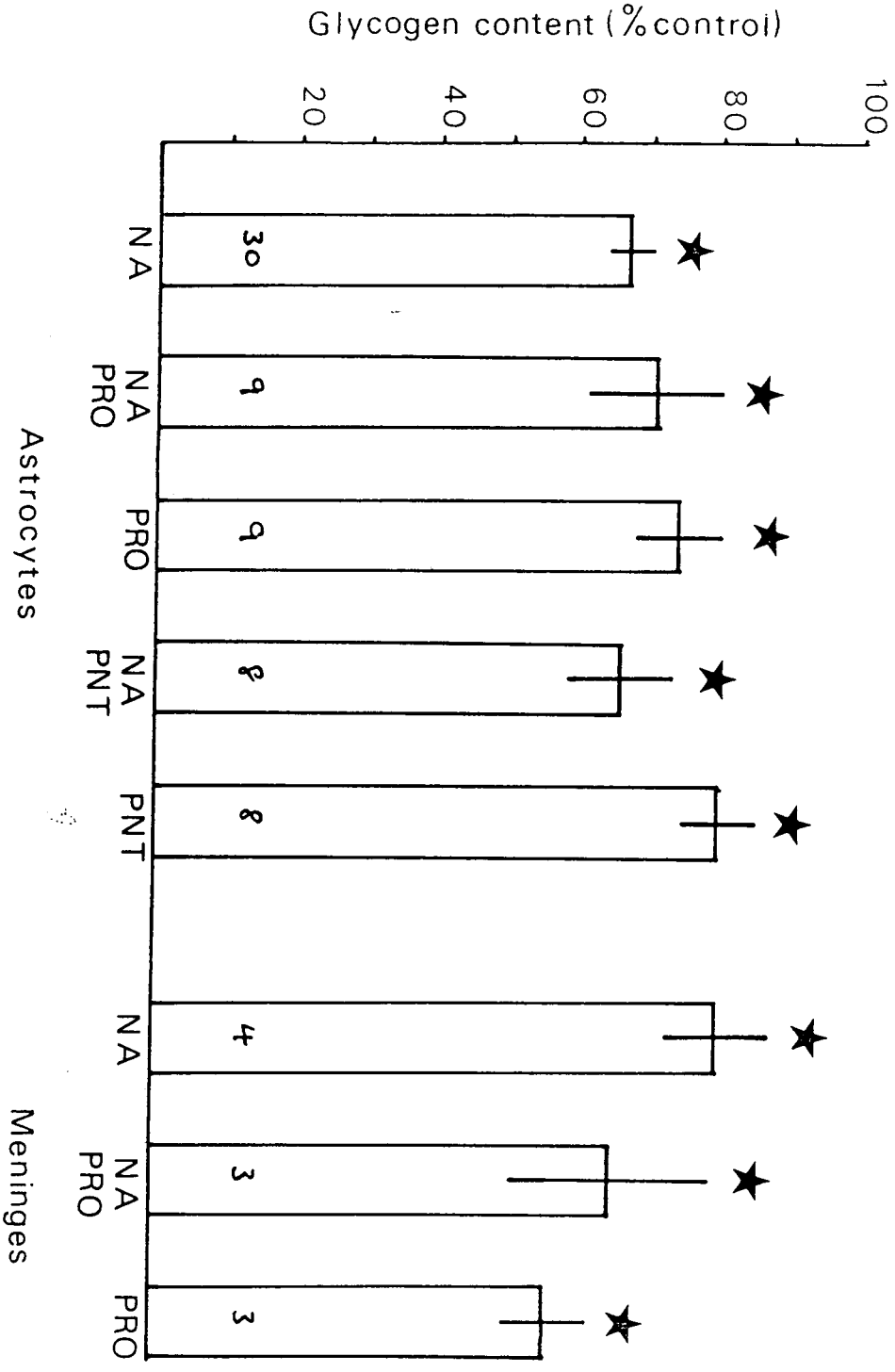
Fig 6.3

Effects of α- and β-adrenoreceptor antagonists on noradrenaline evoked glycogenolysis in cell cultures

Astrocyte-enriched and meningeal cell cultures were incubated with 50 μM noradrenaline (NA) and/or 200 μM propranolol or 200 μM phentolamine and the glycogen content determined as in Methods. Antagonists were added 1-2 minutes before the addition of noradrenaline. n is not less than 6 in each case, the data being derived from the number of experiments shown.

* represents a value statistically significantly different from control ($p<0.05$ or less)

Error bars indicate the standard error of the mean


Glycogen content (% control)
20
40
60
80
100
NA
NA PRO
PRO
NA PNT
PNT
NA
NA PRO
PRO
30
9
9
8
8
4
3
3
Astrocytes
Meninges

in astrocyte glycogen content (20%) and it does not reverse the effect of noradrenaline. The glycogenolytic effect of propranolol is also seen in meningeal cell cultures where it is more marked (44% loss of glycogen).

When α-receptor antagonists selective for α_1 (prazosin, 1 μM) and α_2 (yohimbine, 1 μM) receptor subtypes are used in combination with phenylephrine (Fig 6.4) it is noticeable that neither of these reverses the slight reduction seen in astrocyte glycogen stores after incubation with phenylephrine alone (13%). However, phenylephrine and yohimbine together evoke a 27% decrease in astrocyte glycogen which is statistically significantly less than controls. This effect is not reversed by 1 μM prazosin. Prazosin at this concentration also does not reverse the effect of 50 μM phenylephrine on meningeal cell glycogen (Fig 6.4), although the combination produces a net reduction in glycogen content (23%), close to that loss caused by prazosin alone (20%).

The effects of reserpine on the glycogen content of cell cultures

Fig. 6.5 shows that when astrocyte-enriched cultures are incubated overnight in buffer and reserpine (10 μM) there is a marked statistically significant increase (55%) in glycogen stores over control cultures incubated in buffer alone. However, when meningeal cell cultures are similarly treated there is a statistically significant reduction (19%) in glycogen content.

When astrocyte cultures pre-treated with reserpine are incubated with noradrenaline there is a slight, non-statistically significant increase in the action of noradrenaline on glycogen stores (a 14%

Fig. 6.4

Effects of α_1- and α_2-adrenoreceptor anatagonists on phenylephrine evoked glycogenolysis in cell cultures

Cell cultures were incubated with 50 μM phenylephrine (PHE) and/or 1 μM prazosin (PRAZ) or 1 μM yohimbine (YO) and glycogen content determined as in Methods. Antagonists were added 1-2 minutes before the addition of phenylephrine. n is not less than 6 in each determination, the data being derived from the number of experiments shown.

* represents a value statistically significantly different from control ($p<0.05$ or less)

Error bars indicate the standard error of the mean

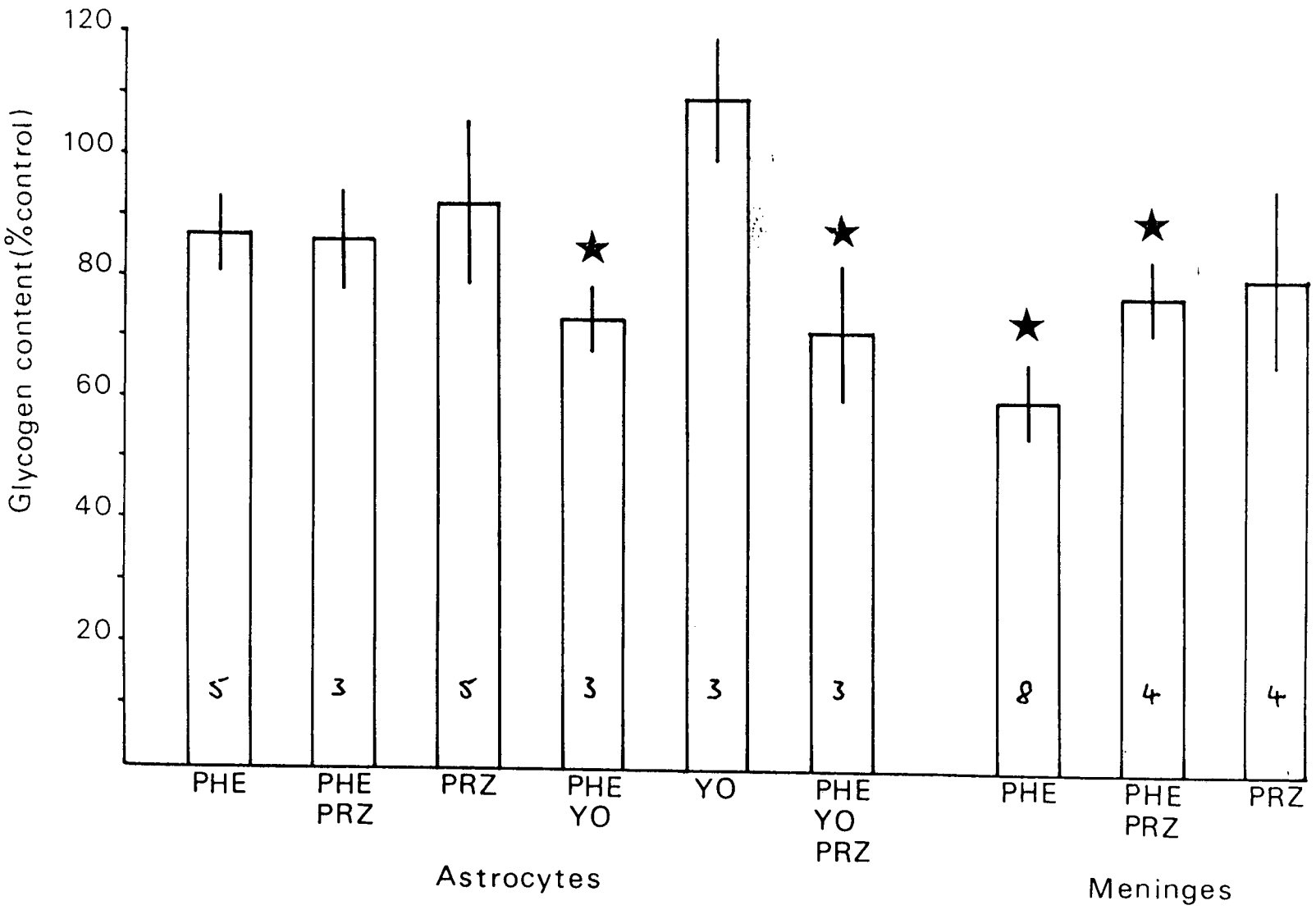

Glycogen content(%control)
120
100
80
60
40
20
5
3
5
3
3
3
8
4
4
PHE
PHE PRZ
PRZ
PHE YO
YO
PHE YO PRZ
PHE
PHE PRZ
PRZ
Astrocytes
Meninges

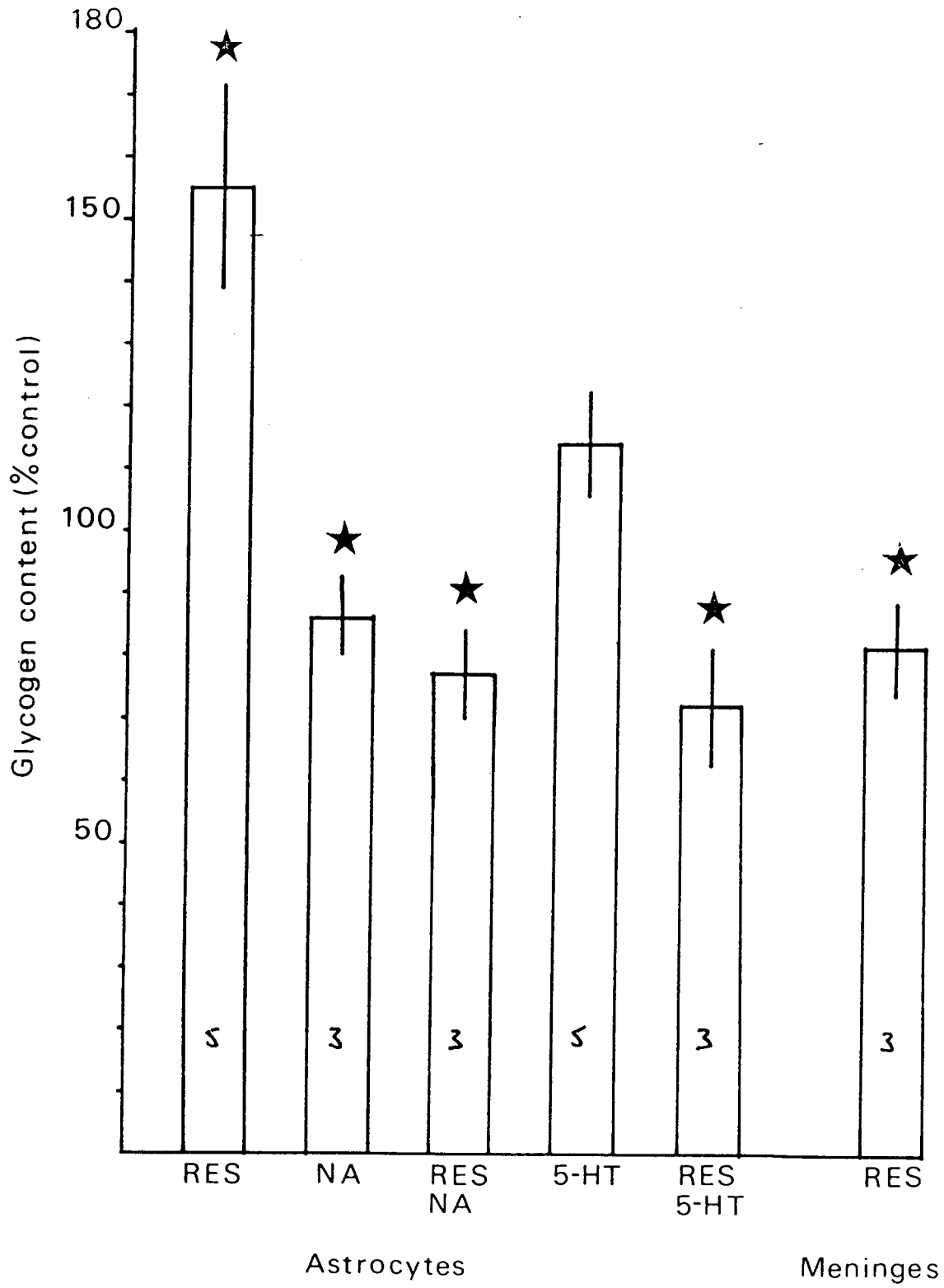
Fig. 6.5

Effects of reserpine on the glycogen content of cell cultures

Cell cultures were incubated with 10 μM reserpine (RES) overnight as described in Methods and the glycogen content determined with or without further exposure to 50 μM noradrenaline (NA) or 50 μM serotonin (5-HT). n is not less than 6 in each case, the data being derived from the number of experiments shown.

* represents a value statistically significantly different from control ($p<0.05$ or less)

Error bars indicate the standard error of the mean


180
150
100
50
Glycogen content (% control)
5
3
3
5
3
3
RES
NA
RES NA
5-HT
RES 5-HT
RES
Astrocytes
Meninges

reduction to a 23% reduction). Moreover, the effect of serotonin on the glycogen stores of non-reserpine treated astrocyte cultures (a 14% increase) is reversed by reserpine pre-treatment to produce a 28% decrease in glycogen which is a statistically significant.

Discussion

The effects of neurotransmitter agonists on the glycogen content of cell cultures

It has previously been suggested that neurons may signal to astrocytes via their release of neurotransmitters. This may result in the modification of the glycogen metabolism of astrocytes thus maintaining a supply of metabolic intermediates for neuronal use. The main aim of the current study was to establish whether stimulation of three neurotransmitter receptor sites, β-adrenergic, serotinergic and muscarinic cholinergic, found on the membranes of astrocytes in vitro could result in a change in the metabolism of astrocyte glycogen stores. The results presented in this section suggest that stimulation of the serotinergic and muscarinic cholinergic receptors is without effect on the net glycogen content of astrocyte cultures. Only noradrenaline is seen to evoke a statistically significant change in astrocyte glycogen stores (a reduction of 33%). Clearly, the smaller effect of noradrenaline on meningeal cell glycogen content (a 20% reduction) is not sufficient to account for the change in astrocyte cultures.

The work of Magistretti et al (1983) has also demonstrated that noradrenaline can evoke glycogenolysis in astrocyte cultures. However, it is notable that the effect of noradrenaline observed by this group (a 60% hydrolysis of [^{3}H]-glycogen) is greater than that

seen here. Moreover, this report also shows that serotonin elicits [^{3}H]-glycogen hydrolysis (37%) in astrocyte cultures. This is not in agreement with the results presented here as it has been shown that serotonin has no statistically significant effect on astrocyte glycogen stores. These discrepancies may be explained in part by the differences between the two methods used to estimate the effects of serotonin on astrocyte glycogen. Magistretti et al (1983) measured the ability of serotonin to hydrolyse [^{3}H]-glycogen newly synthesised by astrocyte cultures from [^{3}H]-glucose. In order for the values of [^{3}H]-glycogen hydrolysis to represent total glycogen breakdown (i.e. the difference in glycogen content of treated and non-treated cells), [^{3}H]-glucose residues and non-labelled glucose residues in [^{3}H]-glycose must be equally available for release by GPa. Glycogen is formed by the addition of the glycosyl group of UDP-glucose to the terminal glucose of an amylose chain (Chapter 4), so [^{3}H]-glycogen will be formed by the addition of [^{3}H]-glucose (and non-labelled glucose) to a pre-existing chain of unknown length. Subsequent hydrolysis of [^{3}H]-glycogen may therefore overestimate the total amount of glycogenolysis. Indeed, if there is a large amount of non-labelled glycogen in relation to [^{3}H]-glycogen, then the glycogenolysis evoked by, for example, serotonin, whilst eliciting marked hydrolysis of [^{3}H]-glycogen, may not be greater than experimental variation when the resultant changes in glycogen content are determined.

Irrespective of these methodological consideration, it is clear that noradrenaline evokes glycogenolysis in astrocyte cultures. Another aim of the current study was to clarify which adrenergic receptor mediates the effects of noradrenaline on astrocyte glycogen stores. As stated in the Introduction, previously published data had suggested that adrenergic control of glycogenolysis in astrocytes

might be mediated by β-receptor, cAMP-linked effects, analogous to the early hypothesis of adrenergic control of hepatic glycogenolysis. Therefore, it might be expected that the noradrenaline evoked net glycogenolysis observed in the astrocyte cultures used here occurs via a cAMP dependent mechanism. Indeed, dBcAMP and also forskolin, (which directly stimulates adenylate cyclase (Seamon et al, 1981) do produce glycogenolysis in astrocyte cultures (38% and 37% respectively). Whilst forskolin also stimulates glycogenolysis in meningeal cells (52%) it is not sufficient to account for the decrease seen in astrocyte cultures (assuming approximately 30% contamination of the total glycogen pool with meningeal cell glycogen). However, whilst increases in intracellular cAMP levels can evoke glycogenolysis in astrocyte cultures it does not necessarily follow that noradrenaline, although known to increase cAMP levels in astrocytes (Chapter 5), elicits glycogenolysis through this mechanism. Notably, the stimulation of astrocyte β-receptors by isoproterenol is also known to increase cAMP in astrocytes (Chapter 5) but it does not produce statistically significant net glycogenolysis (5%) in astrocyte cultures. This is particularly interesting for several reasons:-

i) As there is a marked, statistically significant loss of glycogen in meningeal cell cultures after incubation with isoproterenol (33%) it is unlikely that contaminating meningeal cells contribute significantly to the glycogen pool in astrocyte enriched cultures as previously suggested (Chapter 4).

ii) Increases in intracellular cAMP evoked by transmitter receptor activation do not lead automatically to a net breakdown of astrocyte glycogen.

iii) As isoproterenol (50 μM) stimulates the incorporation of [^{14}C]-2-DG into the glycogen stores of astrocyte cultures (60%; Pearce et al, 1985a), β-receptor activation must therefore also stimulate glycogenolysis, resulting in no net change in glycogen

content, i.e. an increase in turnover rate.

iv) The net glycogen breakdown caused by noradrenaline may not therefore be mediated solely by β-receptors but may involve α-receptors,as is the case in the rat liver where α_1- receptor control of glycogenolysis predominates (Williamson et al, 1981; Kunos, 1984).

However, it is evident that phenylephrine, an α-adrenergic agonist, whilst eliciting a greater reduction in astrocyte glycogen stores (13%) than isoproterenol, does not statistically significantly alter the level of astrocyte glycogen. In contrast, phenylephrine is particularly effective in reducing the level of glycogen in meningeal cell cultures (40%).

These results suggest that, while increased intracellular levels of cAMP can elicit glycogenolysis in astrocyte cultures, this may not be the sole mechanism by which noradrenaline reduces astrocyte glycogen stores. Noradrenaline may evoke glycogenolysis in astrocyte cultures via an action on both α- and β-receptors, unlike the case in meningeal cell cultures where activation of either receptor results in glycogenolysis. In order to examine further the question of whether α- or β-receptors are involved in the action of noradrenaline on astrocyte glycogen stores, selective adrenergic receptor antagonists were used.

Effects of adrenergic antagonists on agonist-evoked glycogenolysis in cell cultures

In view of the inability of a β-receptor agonist (isoproterenol) to elicit net glycogenolysis in astrocytes, the observation that propranolol (a β-receptor antagonist) was ineffective in reversing

noradrenaline-evoked glycogenolysis in these cells was not unexpected. More surprisingly perhaps, propranolol does not alter the noradrenaline-evoked loss of glycogen in meningeal cell cultures which are known to respond to isoproterenol by a net glycogen breakdown. However, propranolol, in the absence of transmitter agonists also causes glycogenolysis in both cell cultures (26% and 44% respectively). This effect may mask any reversal of the action of noradrenaline on both cell cultures. The mechanism by which propranolol reduces glycogen stores is unclear but it is known that it has a variety of effects other than blockade of β-adrenergic receptors. These include blockade of serotonin receptors (Middlemiss, 1984), an increased production of phosphatidic acid (a proposed Ca^{2+} ionophore) in rat brain microsome preparations (Pappu and Hauser, 1983) and an inhibition of ATP evoked Ca^{2+} uptake into rabbit skeletal muscle (Herbette et al, 1983). Therefore, the effects of propranolol on glycogenolysis may be non-specific and involve the intracellular distribution of Ca^{2+}. The importance of Ca^{2+} with respect to glycogen metabolism will be discussed later.

Phentolamine (an α-receptor antagonist) also has effects on the glycogen stores of astrocytes when added in the absence of adrenergic agonists (a 20% reduction) but does not reverse the effect of noradrenaline. As was the case for propranolol, the exact cause of the phentolamine effect is not clear. Thus, phentolamine was of little use as an α-adrenergic antagonist in this case so in order to examine the effects of α-adrenergic stimulation on the glycogen stores of cell cultures, more specific α-adrenergic antagonists were used.

The results from experiments using the α_1-receptor antagonist

prazosin and the α_2-receptor antagonist yohimbine are difficult to interpret. Clearly the small effect of phenylephrine on astrocyte glycogen stores is not reversed by prazosin, suggesting that phenylephrine does not act via an α_1-receptor. This is in contrast to the effect of prazosin on phenylephrine-evoked glycogenolysis in meningeal cell cultures where the level of glycogen in the presence of both agonist and antagonist (77%) is very close to that in the presence of antagonist alone (80% of control). Surprisingly, the effect of phenylephrine and yohimbine (an α_2-receptor antagonist) together on astrocyte glycogen stores, is greater than that of phenylephrine alone (73% and 87% of control respectively). Furthermore, the combination of phenylephrine and yohimbine produces a statistically significant reduction in astrocyte glycogen content. This may suggest that the stimulation of α_2-receptors by phenylephrine may inhibit α_1-mediated, phenylephrine-stimulated glycogenolysis. However, prazosin does not reverse the effect of phenylephrine and yohimbine, a result which contradicts this suggestion.

Phenylephrine is known to potentiate the forskolin evoked stimulation of cAMP accumulation in rat astrocyte cultures via β-receptors (Wu and de Vellis, 1983). It has also been shown to reduce the rises in cAMP evoked by forskolin and β-receptor agonists in astrocyte cultures via α-receptors (Wu and de Vellis, 1983; Evans et al, 1984). It might be suggested that phenylephrine evokes glycogenolysis through stimulation of cAMP (via β-receptors) which is promoted by the inhibition of the antagonistic effects of α_2-receptors by yohimbine. However, presumed increases in cAMP associated with β-receptor stimulation (by isoproterenol) do not stimulate glycogenolysis.

Clearly the action of phenylephrine is complex, and while it is known that α-receptor agonists can inhibit the β-adrenergic agonist evoked rises in cAMP in astrocytes (Evans et al, 1984), nothing is known of the possible interactions of α_1- and α_2-receptor mediated intracellular responses.

The intracellular effects of α_1-receptors in a variety of systems are usually taken to be mediated by stimulation of inositol phospholipid metabolism and effects on intracellular Ca^{2+} distribution (Exton, 1981). Indeed, noradrenaline evokes increases in inositol phospholipid turnover in astrocytes via an α_1-receptor (Pearce et al, 1985c). In contrast α_2-receptor effects are commonly associated with an inhibition of adenylate cyclase (cf. Evans et al, 1984). Notably, an interplay between Ca^{2+} and cAMP has been reported for a variety of systems (see Rapp and Berridge, 1976). Moreover, complex interactions between Ca^{2+} levels and adenylate cyclase have been reported in astrocyte cultures (Brostrom et al, 1976; Ebersolt et al, 1981c; Latzkovits et al, 1982). Therefore, it is possible that the α_2-receptor affects α_1-receptor mediated responses via complex interactions at the level of Ca^{2+} and adenylate cyclase second messenger systems.

Further discussions of such interactions between transmitter receptors and between second messenger systems are beyond the scope of this thesis. However, it is apparent that the effect of noradrenaline on astrocyte glycogen stores may not be mediated solely by β-receptors but may involve complex, obligatory interactions between all three adrenergic receptor sybtypes examined, α_1-, α_2- and β-receptors. In contrast, the action of noradrenaline on meningeal cell glycogen content may be mediated by α_1-receptors (blocked by prazosin) or β-receptors.

The intracellular mechanism(s) by which noradrenaline affects glycogen metabolism in astrocytes subsequent to second messenger production is also not clear. In the liver, adrenergic control of glycogen metabolism is mediated predominantly via α_1-receptors and affects on intracellular Ca^{2+} levels possibly through increases in the turnover of inositol phospholipids (see Introduction, also Hems and Whitton, 1980; Burgess et al, 1984). Ca^{2+} is thought to activate phosphorylase b kinase and thus convert GPb to GPa and so evoke glycogenolysis (Williamson et al, 1981). In cardiac muscle, noradrenaline causes glycogenolysis by activation of both cAMP and Ca^{2+} second messengers which act at different parts of the enzyme cascade then links receptor activation to glycogen breakdown (Rasmussen 1970). Indeed in the liver, cAMP and Ca^{2+}-linked glycogenolytic hormones may act on separate, but overlapping parts of the enzyme cascade (Kunos, 1984).

It has been shown that stimulation of the astrocyte cutlures with selective adrenergic agonists (phenylephrine and isoproterenol) does not evoke net glycogenolysis. This may suggest that noradrenaline acts on astrocyte glycogen stores by an obligatory action on both cAMP and Ca^{2+}. However, as presumed increases in astrocyte cAMP levels not associated with neurotransmitter activation, for example that caused by forskolin, can evoke glycogen breakdown, clearly there must be other control mechanisms involved in receptor activation which influence glycogen metabolism. These may include antagonistic effects of receptors such as that suggested to exist between α_2- and α_1-receptors. Furthermore, as the effects of forskolin and noradrenaline are not additive, there may be a common limiting mechanism of the control of glycogen stores. It is noticeable that the glycogen stores of astrocyte cultures are never reduced to less than

55 - 60% of control (see also Chapter 4) which suggests that there is in fact such a mechanism.

The exact intracellular mechanism(s) by which noradrenaline evokes glycogenolysis in astrocytes is therefore not evident. However, the main aim of this current study was not to determine such mechanisms but to examine the net effects of neurotransmitter agonists on the glycogen content of astrocytes. As a result of this study it is suggested that (a) carbachol and serotonin do not stimulate net glycogen breakdown in astrocytes and (b) the adrenergic control of astrocyte glycogen stores involves both β-adrenergic, cAMP-dependant effects and α-adrenergic, cAMP independant effects and may involve changes in the intracellular distribution of Ca^{2+}, as is believed to be the case in the liver.

The effects of reserpine on the glycogen stores of cell cultures

The anomalous effects of propranolol on the glycogen content of astrocytes have been suggested to be explained by non-specific actions on intracellular Ca^{2+} distribution, implicated in the control of glycogen metabolism in the liver. However, it might also be suggested that noradrenaline is present in the experimental buffer and that propranolol and/or phentolamine act by disturbing the endogenous control of glycogen metabolism, although the possible mechanisms for such effects are far from obvious. There are two possible sources of noradrenaline in the experimental buffer. Firstly, noradrenaline may be derived from the serum present in the culture medium. This seems unlikely as the cell cultures are washed prior to experimental incubations. Furthermore, astrocytes in vitro possess uptake mechanisms for noradrenaline (Kimelberg and Pelton, 1983) and

are capable of metabolising noradrenaline by catechol-o-methyl transferase and both monoamine oxidase A and B (Pelton et al, 1981; Hansson and Sellstrom, 1983; Schoepp and Azzaro, 1983).
The only other possible source of noradrenaline in the cell cultures is from the cells themselves. To examine this hypothesis, the action of reserpine on the glycogen content of astrocytes and meningeal cell cultures was determined.

Reserpine is known to cause a decrease in amine levels in the brain (e.g. Glowinski et al, 1966) by an inhibition of their uptake into neuronal storage vesicles (Shimizu and Ishii, 1964), resulting in subsequent enzymatic breakdown. The reserpine-induced reduction in monoamine neurotransmitter levels is thought to be the reason for the reserpine evoked accumulation of glycogen in the CNS (e.g. Mrsulja and Rosic, 1974) which is observed primarily in astrocytes (Shimizu and Ishii, 1964; Phelps, 1972).

When astrocytes in vitro are treated with reserpine (10 μM) it is seen that they contain subsequently more glycogen (55%) than control astrocyte cultures. The increase in astrocyte glycogen levels caused by reserpine provides evidence that the level of glycogen in astrocyte cultures is controlled by at least one endogenous monoamine neurotransmitter which may act to promote net glycogenolysis. In contrast, reserpine treatment of meningeal cell cultures results in a statistically significant decrease in glycogen content (19%). This suggests that the glycogen metabolism of meningeal cells in vitro may also be controlled by an endogenous monoamine neurotransmitter but in this case the endogenous control promotes net glycogen synthesis.
However, it must be noted that the mechanism of action of reserpine on astrocytes is unknown. Reserpine is generally considered to act by

inhibiting the storage of monoamines in neuronal vesicles by a long-lasting action on vesicular Ca^{2+}/Mg^{2+} ATPase (Sourkes, 1972). Such vesicles have not been reported to occur in cultured astrocytes. Moreover, it has not been shown that astrocytes in vitro contain or synthesise neuroactive amines although dopamine β-hydroxylase, an enzyme which is involved in noradrenaline synthesis has been detected in astrocyte cultures by immunobinding (Murphy, unpublished observations). In addition, it has not been shown that reserpine reduces the level of neuroactive amines in astrocyte or meningeal cell cultures. Some further evidence for the endogenous control of astrocyte glycogen stores is provided by the action of noradrenaline on astrocyte glycogen after pre-treatment with reserpine.

Data from both control and peripheral systems suggest that changes in the affinity and/or density of adrenergic receptors are related to alterations in the availabiltiy of noradrenaline to interact with these receptors (Williams and Lefkowitz, 1978; Bylund, 1979). Indeed reserpine is reported to increase the number of α_1- and β_1-receptors in the brain (U'Prichard and Snyder, 1978) and to cause the appearance of α_2-receptors in rat salivary glands (Bylund and Martinez, 1980). The observed increases in receptor density are matched by an increase in the potency of adrenergic agonists to stimulate second messengers e.g. in the brain reserpine treatment increases the stimulation of adenylate cyclase by noradrenaline and isoproterenol (Dismukes and Dalay, 1974).

Therefore, it might be expected that if noradrenaline is released by cells in astrocyte cultures, then a postulated reduction in noradrenaline levels caused by reserpine might increase the subsequent noradrenaline-stimulated reduction in glycogen stores. In fact this

is shown to be the case, (Fig. 6.5) but the augmentation of the effect of noradrenaline is only slight (8%). This might suggest that noradrenaline is not the endogenous amine controlling the level of glycogen in astrocytes in vitro but might equally suggest that the concentration of noradrenaline used (50 μM) is maximal for stimulation of glycogenolysis irrespective of reserpine pre-treatment.

Two other neuroactive amines have been suggested to affect (reduce) the glycogen content of rodent astrocytes in vitro, namely histamine and serotonin (Magistretti et al, 1983). Under the experimental conditions used in the current study, serotonin does not reduce the glycogen content of astrocyte cultures. However, when serotonin is added to astrocyte cultures pre-treated with reserpine, there is a statistically significant loss of glycogen (27%) compared to control. As the corresponding effect on meningeal cells has not been determined, it must be noted that it is not clear which cell type in astrocyte cultures responds to serotonin. This result suggests that cells in astrocyte cultures (not necessarily astrocytes) release serotonin which in turn regulates the level of glycogen content. Furthermore, as serotonin does not cause net glycogenolysis in astrocyte cultures without pre-treatment with reserpine, the amount of serotonin released may be maximal for glycogenolysis.

The mode of action of serotonin is even less clear than that of noradrenaline. As stated in Chapter 5, a stimulation of cAMP by serotonin in astrocytes has not been established. However, the responses of astrocyte cultures to serotonin with respect to cAMP accumulation may be affected by the presence of endogenously

released serotonin. Fillion et al (1981) have shown that prior exposure of astrocyte isolated from the horse striatum to serotonin reduces the subsequent stimulation of cAMP by this compound. Recent work by Sugino et al (1984) has shown that serotonin increases the level of intracellular Ca^{2+} in C6 glioma. This may indicate a role for Ca^{2+} in serotonin-evoked glycogenolysis in astrocytes.

In conclusion, the main aim of this study was to determine the effects of stimulation of three neurotransmitter receptors known to be present in astrocyte cultures on glycogen metabolism.
A secondary aim was to determine the receptor type which mediates the noradrenergic control of glycogen breakdown in astrocytes. It has been shown that the control of glycogen metabolism in both astrocyte-enriched and meningeal cell cultures is complex. The results from experiments examining the effects of reserpine on the glycogen content of cell cultures provided evidence for the release of one or more monoamine neurotransmitter from both types of culture. Thus any considerations of the mechanisms controlling glycogen metabolism in cell cultures are complicated by several unknown factors. For example, nothing is known of the possible effects of neurotransmitter agonists on the release of endogenous neuroactive amines. These considerations aside, it is apparent that the control of astrocyte culture glycogen content by noradrenaline is unlikely to be mediated by β-receptor, cAMP-dependant effects alone as had previously been suggested or assumed (e.g. Opler and Makman, 1972). In addition, α-receptor, cAMP-independant effects are also indicated to play an obligatory role in such control, possibly through actions on the internal Ca^{2+} concentration of astrocytes.

Irrespective of the exact mechanisms, it is clear that neuro-

transmitters such as noradrenaline and serotonin can influence the metabolism of glycogen in astrocyte cultures. This is consistent with the suggestion that neurotransmitters may act as signals from neurons and modify astrocyte glycogen metabolism.

It has been proposed that metabolic intermediates derived from glycogenolysis may be utilised by neurons (Chapters 1 and 4). In the final chapter, the evidence for such a transfer from experiments performed in our laboratory and others is discussed, together with the future directions of research indicated by the results in this and preceeding chapters.

Summary

1. The effects of various neurotransmitter agonists have been studied on the glycogen stores of astrocyte-enriched and meningeal cell cultures. Only noradrenaline evokes a statistically significant change (a 33% reduction) in the glycogen content of non-reserpine treated astrocytes.

2. Noradrenaline but not carbachol, stimulates glycogenolysis in meningeal cell cultures (20%).

3. Isoproterenol does not stimulate net glycogenolysis in astrocyte cultures (a 5% reduction) whereas it markedly reduces the glycogen content of meningeal cells (33%).

4. Phenylephrine alone does not statistically significantly reduce the glycogen stores of astrocytes (13%) but the addition of yohimbine and phenylephrine evokes a statistically significant reduction (27%) which is not blocked by prazosin.

5. Phenylephrine stimulates net glycogenolysis in meningeal cells (40%) which is substantially reversed by prazosin.

6. Reserpine treatment of astrocyte cultures causes marked changes in the glycogen content (a 55% increase) and in the effect of serotonin (a 28% loss in glycogen stores).

7. Reserpine treatment of meningeal cell cultures causes a reduction in glycogen content (19%).

CHAPTER 7

General conclusions and future directions

The overall aim of this thesis has been to examine the concept that neurons may signal to astrocytes using a variety of means. The work described in the preceeding chapters was performed in order to fulfil a series of aims. First, a model system for astrocytes had to be established in order to examine the effects of putative signals on astrocyte biochemistry. Chapter 2 described attempts to separate astrocytes from the adult rat cortex, which resulted in "astrocyte" fractions unsuitable for further work of the type described here. Primary cultures of astrocytes from the neonatal rat cortex were therefore used as an alternative model for astrocytes in vivo and were extensively characterised with respect to cell types present (Chapter 3).

Secondly, a possible measure of the responsiveness of astrocytes to proposed neuron/astrocyte signals was chosen. Changes in the glycogen stores of astrocytes were taken as such an indicator. This was principally due to the known localisation of CNS glycogen to astrocytes in vivo and the suggested role of glycogen, at least in invertebrate glia, as a store of metabolic intermediates for neurons (Pentreath, 1982).

Thirdly, the nature of suggested signal(s) which may be used by neurons to influence the behaviour of astrocytes was considered. Previous work had shown that astrocytes respond to changes in $[K^+]_o$, e.g. by changes in membrane potential, but moreover, this putative signal had been suggested to influence the metabolism of glycogen in

invertebrate glia (Pentreath, 1982). Thus the effects of changes in $[K^+]_o$ on the glycogen stores of astrocytes were examined (Chapter 4). It was seen that increases in the $[K^+]_o$ over the physiological range (2.5 - 5 mM $[K^+]_o$) evoked reductions in the level of glycogen stores of astrocytes. This suggested that changes in $[K^+]_o$ may represent a signal from neurons to astrocytes, controlling glycogen stores. Glycogen in meningeal cells, used as a control for contaminating cells in astrocyte cultures, was also reduced by increases in $[K^+]_o$ but only at higher concentrations (10 - 15 mM $[K^+]_o$).

Neurotransmitters were also considered as possible neuron/ astrocyte signals. Indeed, binding sites for three neurotransmitter ligands (β-adrenergic, serotonergic and muscarinic cholinergic) were found on membranes prepared from astrocyte cultures (Chapter 5). However, β-adrenergic and serotonergic binding sites were also found on membranes prepared from meningeal cells.

It is evident from experiments described in Chapter 6 that activation of some of the receptors found on membranes prepared from astrocyte and meningeal cell cultures results in complex effects on the glycogen stores of both cell cultures. Adrenergic effects on astrocyte glycogen turnover may involve both α- and β-receptors, both on glycogen synthesis and degradation. The exact mechanism through which noradrenaline causes a net loss of glycogen in astrocyte cultures is unclear. Serotonin also reduces the level of glycogen in astrocyte cultures but only after reserpine treatment. Indeed, reserpine treatment itself causes increases in the level of glycogen in astrocyte cultures. These results suggest that cells in astrocyte cultures may release monoamine neurotransmitters, possibly serotonin.

The control of glycogen stores in meningeal cell cultures by neurotransmitter agonists is also complex but these cells do not contribute large amounts of glycogen to the pool found in astrocyte cultures. However, as shown by the reduction of meningeal cell culture glycogen content caused by reserpine, these cells may also release monoamine neurotransmitters.

The results presented in this thesis indicate areas of future research regarding neuron/astrocyte signalling, not only concerned directly with the responsiveness of astrocytes to neuronal signals with respect to glycogen metabolism but also concerned with broader aspects of neuron/astrocyte interaction. There are many areas of interest regarding the effects of putative signals on astrocyte glycogen stores, some of which are noted here.

The model proposed by Pentreath (1982) suggested that the glycogen stores of glial cells could be utilised by neurons as a reserve store of metabolic intermediates when glucose was not available or was insufficient to maintain neuronal activity. In the experiments described in Chapters 4 and 6, the glucose concentration of the buffer (20 mM) is favourable for net glycogen synthesis in astrocyte primary cultures (Cummins et al, 1983a). In this respect it is notable that in no case in either Chapter 4 or 6 did the stimulus used to evoke glycogenolysis decrease the mean astrocyte glycogen content below 55 - 60% of control. Therefore, it might be proposed that the net loss of astrocyte glycogen elicited by such putative neuron/astrocyte signals, such as an increase in $[K^+]_o$, would be more marked under conditions of reduced external glucose concentration. Similarly, the ability of neurotransmitters to reduce astrocyte

glycogen may also be increased by altering the buffer in this way. Moreover, as it is known that $[K^+]_o$ is increased in vivo during periods of increased neuronal activity, activity which presumably results in the release of neurotransmitters, the effects of neurotransmitter agonists on the glycogen metabolism of astrocytes may also be more marked and more closely represent the case in vivo, under conditions of increased $[K^+]_o$. Similarly, it is known that during periods of neuronal activity, the external Ca^{2+} concentration falls, particularly in the region of the synapse (see Latzkovits et al, 1982). This reduction may in itself represent a signal from neurons to astrocytes and be manifest in alterations of astrocyte glycogen metabolism. Furthermore, the combination of various putative signals such as changes in $[K^+]_o$, neurotransmitters and possible changes in external Ca^{2+} concentration, may result in different effects on astrocyte glycogen metabolism than the individual signals alone. In addition, the responses of astrocytes to putative neuronal signals may also vary with the astrocyte type and location in the brain. In order to examine these suggestions, astrocyte cultures from areas of the CNS other than the cerebral cortex might be used in experiments analogous to those performed here e.g. cerebellum, spinal cord or corpus callosum, where fibrous astrocytes predominate (Peters et al, 1976).

Whilst the experiments already described, and those proposed here for future work, are concerned with possible signalling mechanisms from neurons to astrocytes, they do not examine the effects on astrocyte biochemistry of signals derived directly from neurons. The use of neuron and astrocyte co-cultures and/or of neuronal culture conditioned medium, may therefore be useful in further exploration of the possibility of neuron/astrocyte signalling.

Such experiments might be designed to answer a variety of questions e.g. a) is the glycogen content of astrocyte cultures altered by exposure to neurons or neuron conditioned medium? b) does the ability of neurons to influence astrocyte glycogen stores change during neuronal development? c) do neurons from different parts of the CNS have different effects on astrocyte glycogen stores? d) are any possible effects of neurons on astrocyte glycogen levels altered by stimulation of the neurons with, for example, neurotransmitter agonists? These considerations could overlap to some extent with proposed experiments to examine the effects of possible neuronal signals on different astrocyte sub-types and/or astrocytes derived from different areas of the brain.

The results presented in this thesis might therefore form the basis of extensive further research with respect to the concept of neuron/astrocyte signalling, using changes in astrocyte glycogen stores as an indicator of astrocyte responsiveness to neuronal signals. In addition to these relatively specific suggestions, more broad areas of research are also suggested by the results reported here.

It is evident that the predominant response of astrocyte glycogen to a variety of possible signals is a net reduction in stores. The breakdown of glycogen will initially yield glucose-1-phosphate (through the activity of GPa) and glucose (through the activity of the debranching enzyme). The fate of these products subsequent to their release from glycogen after stimulation by increase in $[K^+]_o$ or, for example, noradrenaline, is totally unknown. However, previous studies have suggested that astrocytes may release a variety of metabolic intermediates for neuronal use. Both Phelps (1972) and Pentreath (1982) suggested that metabolic substrates may be transferred to neurons from astrocytes

as a source of energy for neuronal activity, although no evidence was provided concerning what may be released. However, Selak et al (1984) have recently shown that primary astrocyte cultures release pyruvate into the culture medium. Moreover, these authors state that pyruvate is the major trophic factor in astrocyte conditioned medium which promoted the survival of CNS neurons in vitro. An alternative view of the possible transfer of metabolic intermediates from astrocytes to neurons is given by Shank and Campbell (1984a; b) who suggest that the neurotransmitter pools of glutamate and aspartate in some neurons are replenished by the transfer of α-ketoglutarate and/or malate derived from astrocytes.

Thus a transfer of metabolic intermediates from astrocytes to neurons has been proposed to fulfil a variety of functions, although these are by no means mutually exclusive. However, there is very little published data to support the release of metabolic intermediates from astrocytes and none considering the stimulation or inhibition of this release by putative neuron/astrocyte signals. A major direction of research which is indicated from the work shown in this thesis is therefore to determine whether astrocytes release metabolic intermediates such as glucose or α-keotoglutarate which might be used by neurons. Furthermore, the effects of putative signals from neurons to astrocytes on this release could also be determined.

Current work in our laboratory has shown that astrocytes do indeed release metabolic intermediates, in particular pyruvate, α-keotoglutarate and lactate but not malate (Morrow, unpublished observations). The effects of increased $[K^+]_o$ and neurotransmitter

agonists e.g. noradrenaline on the release of these intermediates are now being determined and may clarify the role of the released intermediates. For example, the release of lactate by astrocyte cultures may reflect only a predominance of anaerobic glycolysis in these cells, as a result of culture conditions, and might not be expected to show a stimulation-evoked release.

The results shown in Chapter 6 provide evidence that cells in astrocyte cultures may release compounds other than metabolic intermediates, i.e. neuroactive monoamines, in particular serotonin. This suggestion may also represent a basis for extensive further research. In particular, as the evidence for the release of serotonin from astrocyte cultures is purely circumstantial(based on the increased effect of serotonin on the glycogen stores of astrocyte cultures) it must be established that monoamine transmitters are released from these cultures. Furthermore, it must be determined from which cells in astrocyte cultures such monoamines are released. It is notable that the effects of reserpine on meningeal cell culture glycogen content (a reduction) suggested that these cells may also release neuroactive amines. Moreover, if astrocytes do release monoamine neurotransmitters, it is conceivable that they may release other neuroactive compounds. It is also possible that the release of neuroactive compounds from astrocytes may be influenced by neuronal signals which may or may not affect astrocyte glycogen metabolism. For example, carbachol has no effect on astrocyte glycogen stores but markedly stimulates the turnover of inositol phospholipids in astrocyte cultures (Pearce et al, 1985c) which in turn may result in increases in the production of arachidonic acid, a precursor of prostaglandins (see Berridge, 1984). In this respect it has recently been shown in our laboratory that astrocyte cultures

synthesise and release prostacyclin, thromboxane and PGE_2 (Murphy et al, unpublished observations). Thus, although changes in glycogen turnover are useful and indeed rare indicators of astrocyte responsiveness to neuronal signals, they may not represent the only feature of neuron/astrocyte interaction. There is a clear need to examine other aspects of astrocyte biochemistry and the possible affects on these of signals from neurons. For example, the synthesis of glutamine in astrocyte cultures (which removes toxic ammonia) is not influenced by the extracellular concentration of ammonia (Yu et al, 1984) but may instead be controlled by signals from active neurons, which may also be producing increased levels of ammonia.

While this thesis has been concerned primarily with the concept of neuron to astrocyte signalling, the possibility that astrocytes may release neuroactive compounds, such as monoamine neurotransmitters, suggests that the reverse may also occur, i.e. that astrocytes signal to neurons. Whether this is indeed the case has yet to be determined. Indeed the possible functions of any signals that might be released from astrocytes are unknown. The neuronotrophic role of factors released from astrocytes is currently the subject of extensive research in other laboratories (cf. Rudge et al, 1985). Notably, serotonin, which may be released from astrocytes, has been proposed to have many trophic functions in the CNS such as the control of neuronal differentiation (see Lauder, 1983). The interactions between neurons and astrocytes may therefore not be a undirectional control of astrocyte biochemistry by neurons, but be a complex, and dynamic, two-way process.

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